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Deprivation and Radiation Via Manipulation of the MDM2
Pathway

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| 13. ABSTRACT (Maximum 200 Words) Androgen deprivation (AD) is a common treatment for prostate cancer, yet the mechanisms of action are poorly understood. Radiotherapy (RT) is also often used in the treatment of localized disease and AD+RT in more high risk cases. Our results indicate that MDM2 is central to prostate cancer response to AD, RT, and AD+RT. Our data establish that by reducing the expression of MDM2 with an antisense oligonucleotide (AS-MDM2) the apoptotic response of LNCaP cells in vitro to AD, RT, and AD+RT is increased. The enhancements in apoptosis translated into gains in overall cell death (measured by clonogenic assay). These interactions were reduced in LNCaP-MST cells that overexpressed MDM2. Preliminary findings in vivo indicate that AS-MDM2 sensitizes LNCaP cells to AD; the experiments are still in progress. We have also found that MDM2 overexpression was evident in 48% of a cohort of locally advanced men treated on Radiation Therapy Oncology Group protocol 86-10, correlated with Gleason score and was associated with a trend for increased distant metastasis. MDM2 is a promising target for enhancing prostate cancer response to AD, RT, and AD+RT, which could potentially impact men with virtually any stage of disease. | | | | |
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INTRODUCTION

Androgen deprivation (AD) continues to be one of the most common treatments for prostate cancer; yet, the mechanisms of action are poorly understood. Normal prostate epithelial cells are induced into apoptosis in over 80% within 10 days of AD.¹⁻³ In contrast, the apoptotic response of prostate tumor cells occurs in the minority, with the majority induced into a resting state.⁴⁻⁹ In order to take full advantage of AD as a therapeutic modality, the mechanisms responsible for the diminished apoptotic response of tumor cells must be elucidated. Our data indicate that MDM2 has a central role in this process; antisense-MDM2 (AS-MDM2) restores the apoptotic response of prostate cancer cells to AD.

Prostate cancer responds to radiotherapy (RT), which is an important treatment modality for men without distant metastasis. In those with high-risk features, local relapse is relatively common. The apoptotic response of prostate cancer cells to radiation is minimal and most cell death appears to be mitotic. Our data show that MDM2 increases the apoptotic and overall cell death responses of prostate cancer cells to RT.

The scope of this research is that through manipulating MDM2 expression via AS-MDM2 it may be possible to improve the efficacy of RT, AD and the combination. Virtually any patient, from those with early disease to those with distant metastasis, stand to benefit.

BODY

Task 1. Determine the impact of MDM2 suppression and overexpression on the interaction of AD and RT in promoting cell death and inhibiting prostate cancer growth in vitro and in vivo.

- a. Complete in vitro apoptosis measurements on LNCaP cells treated in vitro with AS in combination with AD, RT, and AD+RT. Months 1-6.
- b. Baseline cell viability, cell number apoptosis, and clonogenic assays of LNCaP-MST. Months 1-6.
- c. Time course experiments of AS effects on AD, RT, and AD+RT in LNCaP and LNCaP-MST cells. Months 7-12.
- d. In vivo experiments of the action of AS on LNCaP and LNCaP-MST cells. Months 7-36

We have completed components a-c in Task 1 and the results have been presented at two meetings^{10, 11} and published in two papers.^{12, 13} To summarize the AD results¹³, AS-MDM2 was effective at promoting cell death in LNCaP cells by apoptosis using a caspase 3+7 assay and an Annexin V assay, as compared mismatch (MM) controls. AD alone had little influence on apoptosis, but when AS-MDM + AD were given together there was a supra-additive enhancement in apoptosis. When the synthetic androgen R1881 was added to AS-MDM2+AD the apoptotic responses were significantly reduced. The increase in LNCaP cell killing by apoptosis translated into an overall increase in cell death, as determined by clonogenic cell survival assay. The interaction of AS-MDM2 with AD was much less pronounced in LNCaP-MST cells, which overexpress MDM2.

AS-MDM2 also resulted in an increase in apoptosis and clonogenic cell survival when added to LNCaP cells exposed to single fraction RT.¹² Further enhancement in cell death via apoptosis was consistently observed when AS-MDM2+AD+RT was compared to AD+AS-MDM2 or AS-MDM2+RT, although the differences weren't always significant. However, a

significant increase in cell death by clonogenic survival was observed with AS-MDM2+AD+RT, as compared to AD+AS-MDM2 or AS-MDM2+RT. These data demonstrate the key role of MDM2 in the apoptotic responses of the AD and RT, and that the use of AS-MDM2 has tremendous potential as an adjunct to AD and RT for the treatment of prostate cancer.

Table 1

| Group | Animal Number | Number of PSA Non-Failures* | Percentage of PSA Non-Failures |
|---------------|---------------|-----------------------------|--------------------------------|
| AS-MDM2 Alone | 12 | 0 | 0% |
| AD Alone | 4 | 1 | 25% |
| AS+RT | 16 | 3 | 19% |
| AS+AD | 13 | 4 | 31% |
| AS+AD+RT | 16 | 5 | 31% |

*Serum PSA <1.5 ng/ml at 6 weeks

Section d of Task 1 concerns the preliminary in vivo studies of AS-MDM2 in LNCaP cells grown in nude mice. These studies are in progress (**Table 1**) We are currently treating with AS-MDM2 (25 mg/kg per injection) for 10 days and administering 5 Gy RT as a single fraction on day 5 in the middle of the AS-MDM2 injections. AD is initiated 3 days prior to the start of the AS-MDM2. We have begun to increase the AS-MDM2 injections to 15 days to see if the response is enhanced. Most of the effects that we are seeing preliminarily appear to be between AS-MDM2 and AD, although the numbers are small. We are also performing tumor volume cell kinetic studies using small animal MRI.

We have developed micro-MRI techniques for the visualisation of LNCaP tumors grown orthotopically in the prostates of nude mice. Imaging these tumors presented a series of unique challenges. Unlike most tumors, they do not appear hyper-intense on T₂ weighted images, and apparently do not exhibit the leaky vasclature that makes tumors easily detected with small molecule contrast agents. However, by making images of the highest spatial resolution achievable with our 7-tesla micro-MRI system, we have developed a protocol which permits clear visualization of the anatomy.

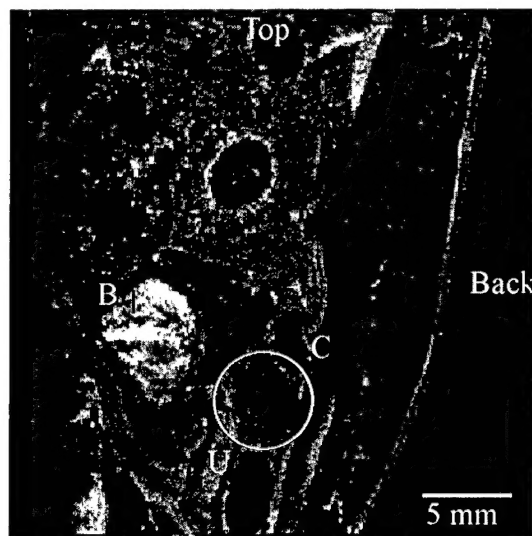
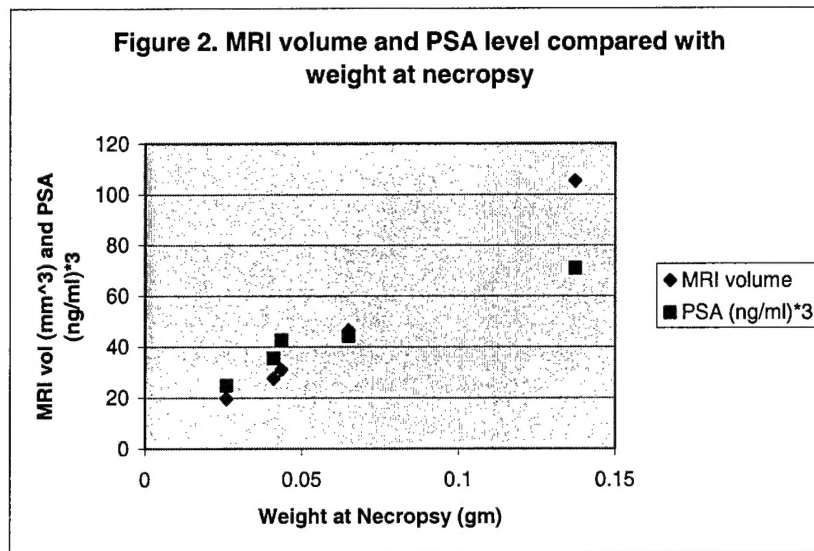


Figure 1. MRI of A Tumor-Bearing Mouse

Shown at left is a sagittal section taken from a three-dimensional micro-MRI dataset. The tumor is circled in yellow. As can be seen from the scale bar in the lower right hand corner, the tumor is approximately 5 mm in diameter on this section. Also labelled are the bladder (B), the colon (C), and the urethra (U). The image represents one section of a three dimensional data set, with isotropic voxel size of 0.1mm. Tumor volumes are measured by manually outlining the tumors on a series of slices, and summing the volumes of the individual slices. The tumors are identified as anomalous structures that form between the urethra and colon, and are located slightly inferior to the bladder. The images were made with a gradient

echo pulse sequence, and the MRI acquisition parameters were T_R=200msec, T_E=3.3msec, field

of view = 2.56x2.56x1.128 cm. The animal receives an injection of Gd-DTPA (2ml Magnevist), diluted 10:1 in saline) prior to the scan.



In the graph, tumor volume (mm³) and PSA level (ng/ml, multiplied by a factor of three to facilitate the comparison with our volume measurements) are plotted as a function of tumor weight at necropsy for five animals. These results demonstrate that our non-invasive measurements of tumor volume agree well with both PSA and tumor weight at necropsy, indicating that we can use MRI for monitoring

tumor response to treatment

Task 2. Define the molecular mechanisms underlying the changes in LNCaP cell killing in response to AD ± RT when MDM2 is suppressed or overexpressed.

- Western blot analysis of p53, p21, MDM2, bcl-2, bax, E2F-1 and pRB under conditions of AD and AS given simultaneously. Months 1-12.
- Western blot analysis of p53, p21, MDM2, bcl-2, bax, E2F-1 and pRB under conditions of AD given 2 d before AS. Months 13-24.
- Manipulation of gene expression to further enhance/replace the action of AD, RT, or AD+RT based on the Western results from the studies in years 1 and 2; for example, targeting p53 using adenoviral-p53, E2f-1 using adenoviral E2F-1, or bcl-2 using antisense bcl-2. Months 25-36

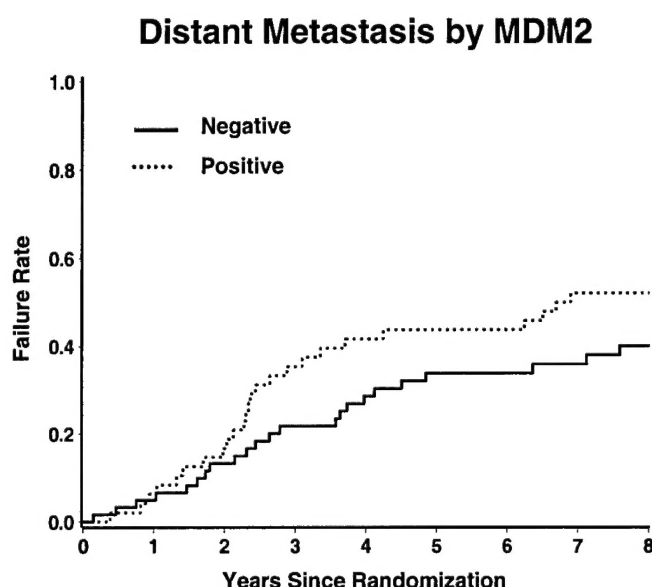
A significant proportion of the proposed LNCaP Western blot analyses were included in the two papers that were described above.^{12, 13} The analyses included the protein levels of MDM2, p53, and p21. AS-MDM2 caused a reduction in MDM2, which was even further reduced by AD. P53 and p21 increased after AS-MDM2 or RT. We have also looked at bcl-2 and bax levels in response to AS-MDM2, and have not found much of an effect. We are exploring the use of measuring RNA gene expression in cDNA microarrays to obtain a more complete understanding of the genes affected by AS-MDM2, AD and RT.

Task 3. Examine the degree and predictive value of MDM2 overexpression in diagnostic archival tissue specimens from patients treated with RT alone and RT + AD.

- MDM2 immunohistochemistry analysis of 110 cases from RTOG protocol 86-10. Months 1-6.
- Statistical analysis of MDM2 staining results from RTOG protocol 86-10. Months 7-10.

- c. MDM2 immunohistochemistry analysis of cases from RTOG protocol 92-02. Months 7-30.
- d. Statistical analysis of MDM2 staining results from RTOG protocol 92-02. Months 30-36.

Figure 3. Distant Metastasis By MDM2 Expression



The immunohistochemical staining and analysis of MDM2 expression in 109 diagnostic samples from patients treated in RTOG 86-10 has been completed and an abstract on the results submitted (American Society of Therapeutic Radiation Oncologists annual meeting, October, 2004). The nuclear staining of MDM2 expression was quantified manually and using an image analysis system (ACIS, ChromaVision, San Juan Capistrano, CA). By manual count determination, MDM2 overexpression (defined as >5% of tumor cells with nuclear staining) was seen in 48% of the samples and MDM2 overexpression was significantly related to higher Gleason score. Similar results

have been reported by Leite et al.¹⁴ An extensive analysis was performed to determine if MDM2 overexpression was associated with local failure, distant metastasis, cause specific mortality, and overall mortality in univariate and Cox proportional hazards multivariate analyses. None of these relationships were significant, but a pattern of increased distant metastasis was observed (Figure 3).

Table 2

| MDM2 ACIS Count | N | Failures | RR* | (95% CI) | p-value [†] |
|--------------------|----|----------|------|--------------|----------------------|
| ≤1.0% | 28 | 13 | 1.05 | (0.56, 1.96) | 0.88 |
| >1.0% | 80 | 41 | | | |
| ≤3.0% | 59 | 26 | 1.69 | (0.98, 2.91) | 0.057 |
| >3.0% | 49 | 28 | | | |
| ≤5.0% | 82 | 39 | 1.28 | (0.70, 2.34) | 0.42 |
| >5.0% | 26 | 15 | | | |

*RR = relative risk; CI = confidence interval

MDM2 overexpression and distant metastasis; however, MDM2 expression as determined by ACIS counts was not a significant predictor of distant metastasis or any of the other endpoints tested in multivariate analysis.

Although the expression of MDM2 was not independently predictive of distant metastasis, the findings in univariate analysis are encouraging and provide a rationale for

We then quantified the proportion of tumor cells that had nuclear staining above background using the ACIS image analysis system. As shown in **Table 2**, at the 3% cutpoint, which was the median value, there was a borderline significant relationship between

continued exploration of this marker in a larger cohort of patients treated with RT+AD. With larger patient numbers, MDM2 overexpression may emerge as a significant covariate. In addition, MDM2 overexpression may be more discriminating in men with earlier stage prostate cancer. The patients in RTOG protocol 86-10 were so locally advanced that we rarely see such patients in the clinic today. We are in the process of staining the samples from RTOG 92-02, which is comprised of patients that are high risk, but with features more consistent with the contemporary high-risk patient. There are over 500 cases with tissue for MDM2 staining from RTOG 92-02, so there will be much greater power to detect a difference.

KEY RESEARCH ACCOMPLISHMENTS

- AS-MDM2 sensitizes LNCaP prostate cancer cells to androgen deprivation, radiation and the combination. This is the first study to demonstrate such an interaction.
- Apoptosis appears to be the major cell death pathway affected by AS-MDM2.
- MDM2 overexpressing LNCaP-MST cells were more resistant to the sensitizing action of AS-MDM2, confirming the role of MDM2 in the development of prostate cancer cell resistance to androgen deprivation.
- AS-MDM2 increases p53 and p21 expression, demonstrating that the negative feedback pathway was intact.
- MDM2 overexpression is seen in 48% of men with locally advanced prostate cancer (samples from RTOG protocol 86-10) and is associated with a trend for increased distant metastasis. This is the only study to examine the relationship of MDM2 expression to patient outcome after radiotherapy with or without androgen deprivation.

REPORTABLE OUTCOMES

1. **Pollack A**, Mu Z, Hachem P, Agrawal S. "Modulation of Prostate Cancer Cell Death in Response to Androgen Deprivation and Radiation by MDM2. International Conference on Translational Research 2003, Lugano, Switzerland 3/17/03
2. Mu Z, Hachem P, Agrawal S, **Pollack A**. Antisense MDM2 sensitizes prostate cancer cells to androgen deprivation, radiation, and the combination. *Int J Radiat Oncol Biol Phys* 2003;57:S256-257.
3. Mu Z, Hachem P, Agrawal S, **Pollack A**. Antisense MDM2 sensitizes prostate cancer cells to androgen deprivation, radiation, and the combination. *Int J Radiat Oncol Biol Phys* 2004;58:336-343.
4. Mu Z, Hachem P, Sudhir A, **Pollack A**. Antisense MDM2 Oligonucleotides Restore the Apoptotic Response Of Prostate Cancer Cells To Androgen Deprivation. *The Prostate* 2004;In-Press.

CONCLUSIONS

The in vitro results strengthen our initial hypothesis that MDM2 is a key factor in the apoptotic and overall cell death responses of prostate cancer cells to AD and RT. The in vivo studies that are in progress seem to support the in vitro findings, at least for an interaction between MDM2 and response to AD. AS-MDM2 holds promise as a therapeutic strategy for nearly every group of men with prostate cancer. Those with favorable to intermediate risk localized disease may benefit from the use of lower doses of RT and consequently reduced side effects. Those with high-risk localized disease are usually treated with AD+RT and have a

significant risk of microscopic nodal and distant metastasis; AS-MDM2 would act to enhance the effect of RT locally and AD systemically.

Nuclear MDM2 overexpression by immunohistochemical staining was found to be associated with an increased risk of distant metastasis in men enrolled in RTOG protocol 86-10, although the association was not statistically significant. The participants in this study had very advanced disease and are not representative of those with high-risk features that are seen more routinely for treatment today. The lack of a correlation between MDM2 expression and local failure in archival samples from RTOG protocol 86-10 may also be because this parameter is not a very reliable indicator of eradication of the disease locally. Even though this study was initiated in the pre-PSA era, PSA undoubtedly had a role in follow-up. The early identification of failure by PSA and identification of distant spread would have precluded attempts to confirm local persistence of disease by prostate biopsy. For these reasons, it is reasonable to pursue the staining of MDM2 in RTOG protocol 92-02.

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APPENDICES

There is one abstract and two papers

or in combination were evaluated using clonogenic, M.T.T., and cells numeration assays. In addition, cell cycle analysis using flow cytometry were performed to approach the mechanism of action of our experimental findings.

Results: Treatment with Letrozol in MCF7-3 (2) shuttled down the measured aromatase activity to the control cells level. Cells numeration assay showed an inhibition of 50 and 75% of the growth of the MCF7-3 (2) when Letrozol is used alone for more than 6 days at 0.7 μ M and 7nM, respectively. M.T.T. assay confirmed these first results. When radiation therapy was used alone, survival fraction decreased in a dose-dependant manner and survival curves were fitted using a linear-quadratic model. Survival fractions at 2 Gy (SF2) were 0.65 and 0.53 with radiation alone and radiation plus Letrozol, respectively ($p=0.001$). At 4 Gy, survival fractions were also statistically higher with radiation alone (0.56) than with radiation plus Letrozol (0.09, $p=0.0001$). Growth of MCF7-3 (2) was decrease by 76% (cells numeration) six days after radiotherapy (2 and 4 Gy) and Letrozol (0.7 μ M) treatment as compared to radiotherapy alone. This growth reduction of the MCF7-3 (2) cells was higher to 85% after twelve days of the same treatment combination. M.T.T. assay confirmed these results at 2 and 4 Gy in combination with Letrozol (0.7 μ M). After fifteen days of Letrozol (0.7 μ M) alone, cell cycle analyses showed a G1 arrest (77%) and a decrease of the S phase (13%) as compared to control cells, 63% and 27%, respectively. In combination treatment, as compared with radiation alone, we observed a decrease of the radio-induced G2 phase arrest (11% vs 15%), cell redistribution in the G1 phase (72% vs 62%), and a decrease of the S cell cycle phase (17% vs 23%).

Conclusions: Letrozol is an active cytostatic or cytotoxic agent used alone in breast cancer transfected cell lines. In combination with radiation therapy, Letrozol has been proved to be a powerful radiosensitizer. These radiobiological results are the first pre-clinical findings of radiosensitization of Letrozol and will be further basis for adjuvant therapeutic combination in clinical settings.

216 Antisense MDM2 Sensitizes Prostate Cancer Cells to Androgen Deprivation, Radiation, and the Combination

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Purpose/Objective: Antisense MDM2 (AS) sensitizes a variety of tumor cell types, including prostate cancer, to radiation and chemotherapy. We have previously described that AS enhances the apoptotic response to androgen deprivation (AD) and that this translates into a reduction in overall cell survival, as measured by clonogenic assay. Since AD+RT is a key strategy for the treatment of men with high risk prostate cancer, AS was tested for the ability to sensitize cells to the combination of AD+RT.

Materials/Methods: LNCaP cells were cultured in vitro in either complete medium, charcoal stripped androgen deprived (AD) medium, or AD medium supplemented with the synthetic androgen R1881 (10⁻¹⁰ M) for 2 to 3 days. AS was then administered for 18 to 24 hr before RT was given. Processing of the cells after RT was done at 3 hr for Western blots, 24 and 48 hr for trypan blue dye exclusion, 18 hr for Annexin V staining and flow cytometric analysis, 18 hr for caspases 3+7 quantification by fluorometric assay and immediately for clonogenic survival measured 12 to 14 days later. There were 18 treatment groups that were studied: Lipofectin control (LC), AS, antisense mismatch (ASM), AD, AD+R1881, AD+AS, AD+ASM, AD+AS+R1881, AD+ASM+R1881, RT, RT+AS, RT+ASM, RT+AD, RT+AD+R1881, RT+AS+AD, RT+ASM+AD, RT+ASM+AD+R1881, RT+AS+AD+R1881. Statistical comparisons between groups were accomplished with one-way analysis of variance using the Bonferroni test, considering all 18 groups.

Results: AS caused a reduction in MDM2 expression and an increase in p53 and p21 expression. Early cell death by trypan blue was found to be reflective of the apoptotic results by Annexin V and Caspase 3+7. AS caused a significant increase in apoptosis over the LC, AD and RT controls. Apoptosis was further increased significantly by the addition of AD or RT to AS. When AS+AD+RT were combined there was a consistent increase in early cell death over AS+AD and AS+RT by all of the assay methods, although this increase was not significant. Overall cell death, as measured by clonogenic assay, revealed synergistic cell killing of AS+RT (2, 4, and 6 Gy) beyond that of ASM+RT and LC (no oligonucleotide) +RT, and AS+RT+AD over ASM+RT+AD, RT+AD, ASM+RT+AD+R1881 and AS+RT+AD+R1881 (See Figs 1A/B).

Conclusions: AS sensitizes cells to AD, RT, and AD+RT, and shows promise in the treatment of the full range of patients with prostate cancer. AS has the potential to sensitize the primary tumor to RT and metastasis to AD.

Figure 1A

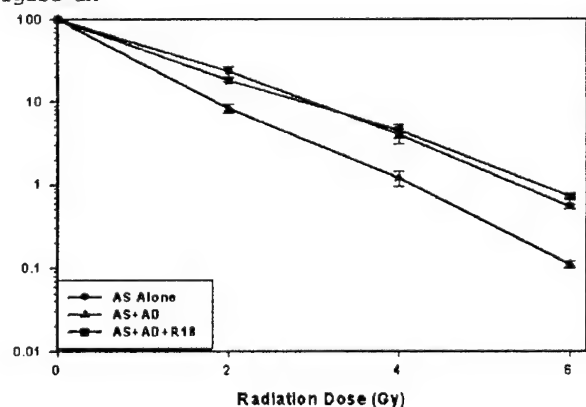
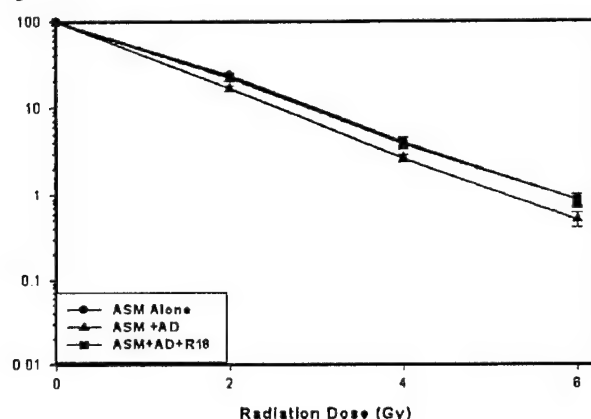


Figure 1B



217 Increased Cure Rate of Glioblastoma Using Concurrent Therapy with High Dose Radiation and Arsenic Trioxide

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Purpose/Objective: Patients with glioblastoma (GBM) do extremely poorly despite aggressive therapy with surgery, radiation therapy and chemotherapy. In an effort to increase the efficacy of therapy for GBM, we studied the efficacy of arsenic trioxide (ATO) combined with high dose radiation therapy in GBM cells in vitro and in GBM xenograft tumors in nude mice. Studies were designed to take advantage of the ROS generating capability of ATO as a potential radiosensitizer, and the use of a large single dose of radiation (similar to that used in stereotactic radiosurgery) to break down the blood-brain barrier in order to optimize the potential effects of the ATO intracranially.

Materials/Methods: Human glioblastoma cell line SNB75 cells were irradiated in vitro with doses of 0–15 Gy with or without ATO. Clonogenic assay was used to generate the radiation survival curves. Antitumor efficacy of the combined therapy was tested in nude mice bearing established subcutaneous SNB75 tumors. A single dose of 20 Gy radiation was administered locally. A single dose of ATO at 10 mg/kg was injected i.p. 10 minutes after irradiation.

Results: Human GBM SNB75 cells were irradiated in vitro with doses of 0–15 Gy, with or without ATO. Radiation survival curves demonstrated that a dose of 0.2 μ M ATO increased radiation-induced cell killing by 2 logs at 10 Gy. ATO at 1 μ M decreased survival from 4×10^2 following 7-Gy radiation alone to 4×10^0 . A time course experiment demonstrated that the highest level of cell killing occurred when ATO was administered immediately before or within 2 hours following irradiation. To test the therapeutic efficacy of this combined treatment regimen in vivo, nude mice with established SNB75 GBM tumors were treated with a single local tumor dose of 20Gy radiation therapy with or without a single dose of ATO at 10 mg/kg. Appropriate control groups were included as well. ATO alone did not inhibit or delay tumor growth. 20 Gy radiation therapy alone inhibited tumor growth by 45 days, with regrowth of tumors thereafter. The combination treatment of radiation therapy and ATO resulted in complete regression of the tumors in 4 out of 5 mice without tumor regrowth out to 3 months. One mouse in the combined treatment group had a 90% reduction in tumor size without progression during the 3 months follow up period. Furthermore, ATO alone and in combination with irradiation did not produce any obvious signs of toxicity (e.g. general appearance or activity level of the mice and no significant loss of body weight).

Conclusions: These results demonstrate that radiation therapy combined with ATO is an effective treatment for GBM tumors in mice. These preclinical results are encouraging and provide a rationale for further study of ATO combined with radiation for the treatment of GBM and other histological types of brain cancer using a variety of radiation therapy schemes, with consideration given to the initiation of clinical trials using this therapy in patients with brain tumors in the near future.

218 Inhibition of HU177 Cryptic Epitope Within Collagen Enhances the Antiproliferative Effects of Ionizing Radiation in B16 Melanoma

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Purpose/Objective: Proteolytic remodeling of the extracellular matrix (ECM) plays a critical role in regulating both angiogenesis and tumor invasion. A panel of monoclonal antibodies against cryptic epitopes of collagen, selectively exposed during extracellular matrix (ECM) remodeling, has been generated by subtractive immunization. One of these cryptic epitopes, termed HU177, is expressed selectively during proteolysis or denaturation of collagen. We previously reported that radiation therapy modulates the exposure of the HU126 cryptic epitope recognition but it does not hinder the exposure of HU177 (*IJROBP* 2002;54(4):1194–1201). In several preclinical models, treatment with monoclonal antibody (Mab) directed to the HU177 epitope was shown to inhibit both angiogenesis and tumor growth in vivo. We sought to examine the effects of Mab HU177 on B16 murine melanoma tumor growth in vivo and to evaluate whether blocking the HU177 cryptic epitope can enhance the effects of ionizing radiation in vitro.

Materials/Methods: a) **In vivo Murine Model:** BALB/C mice were injected subcutaneously into the right flank with 1×10^6 syngeneic B16 melanoma cells. Thirty-eight mice were randomly assigned to the following four groups: control(no

ANTISENSE MDM2 SENSITIZES PROSTATE CANCER CELLS TO ANDROGEN DEPRIVATION, RADIATION, AND THE COMBINATION

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Purpose: Antisense MDM2 (AS) sensitizes a variety of tumor cell types, including prostate cancer, to radiation and chemotherapy. We have previously described that AS enhances the apoptotic response to androgen deprivation (AD) and that this translates into a reduction in overall cell survival, as measured by clonogenic assay. Because AD + radiation (RT) is a key strategy for the treatment of men with high-risk prostate cancer, AS was tested for the ability to sensitize cells to the combination of AD+RT.

Methods and Materials: LNCaP cells were cultured *in vitro* in either complete, androgen deprived (AD), or AD+R1881 (synthetic androgen) medium for 2–3 days before AS was administered. Radiation at 5 Gy was given 18–24 h later. Processing of the cells after RT was done at 3 h for Western blots, 24 and 48 h for trypan blue dye exclusion, 18 h for Annexin V staining by flow cytometric analysis, 18 h for Caspase 3+7 quantification by fluorometric assay, and immediately for clonogenic survival measured 12–14 days later. There were 18 treatment groups that were studied: lipofectin control, AS, antisense mismatch (ASM), AD, AD+R1881, and RT in all possible combinations. Statistical comparisons between groups were accomplished with one-way analysis of variance using the Bonferroni test, considering all 18 groups.

Results: AS caused a reduction in MDM2 expression and an increase in p53 and p21 expression. Early cell death by trypan blue was found to be reflective of the apoptotic results by Annexin V and Caspase 3+7. AS caused a significant increase in apoptosis over the lipofectin control, AD, and RT controls. Apoptosis was further increased significantly by the addition of AD or RT to AS. When AS, AD, and RT were combined, there was a consistent increase in early cell death over AS+AD and AS+RT by all of the assay methods, although this increase was not significant. Overall cell death measured by clonogenic assay revealed synergistic cell killing of AS+RT beyond that of ASM+RT and RT alone, and AS+RT+AD beyond that of AS+RT, AS+RT+AD+R1881, ASM+RT+AD, and ASM+RT+AD+R1881.

Conclusion: AS sensitizes cells to AD, RT, and AD+RT and shows promise in the treatment of the full range of patients with prostate cancer. AS has the potential to sensitize the primary tumor to AD+RT and metastasis to AD. © 2004 Elsevier Inc.

Antisense, MDM2, Androgen deprivation, Radiation, Prostate cancer.

INTRODUCTION

The combination of androgen deprivation (AD) plus radiation (RT) has become the standard for patients with high-risk prostate cancer. Despite the documentation of a survival improvement from this combination over RT alone in some series (1–3), there are still questions regarding the long-term efficacy over AD alone (4). An understanding of the molecular events that occur in the response of cells to AD and RT could lead to novel strategies that enhance cell killing in response to these agents, thereby allowing for the potential

to reduce toxicity through reduced exposure. It may be possible even to replace AD and RT altogether with less morbid alternative biologic therapies. Our approach has been to manipulate the apoptotic pathway.

Recently, we focused on MDM2 as a target for enhancing the apoptotic response of LNCaP cells to AD. The rationale was that MDM2 is overexpressed in 30–40% of prostate cancers (5, 6), MDM2 regulates p53 expression through a negative feedback loop (7), and p53 has been implicated in the apoptotic response of prostate epithelial cells to AD (8–12). An effective method for ablating MDM2 expression

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is through antisense MDM2 (AS) (13–16). Prior studies from our group have shown that AS+AD results in increased apoptosis over that seen by AS, AD, antisense mismatch (ASM), or ASM+AD (17). The pattern of increased early apoptotic cell death was mirrored in clonogenic survival assays, suggesting that overall cell death of LNCaP cells was significantly enhanced by the addition of AS to AD. Because AS has been shown to sensitize cells to RT and chemotherapy in a number of cell lines, it was hypothesized that AS will sensitize prostate cancer cells not only to AD and RT given individually, but also to AD+RT. Wild-type p53-expressing human LNCaP cells were chosen for the investigation of the effects of AS on AD+RT.

METHODS AND MATERIALS

Antisense oligonucleotides

The oligonucleotides were provided by Hybridon, Inc. (Cambridge, MA). The antisense MDM2 oligonucleotide (AS) and its mismatch control oligonucleotide (ASM) are 20-mer mixed-backbone oligonucleotides with following sequence (AS; 5'-UGACACCTGTTCTCACUCAC-3') and (ASM; 5'-UGTCACCCTTTTCATUCAC-3'). They were stored as frozen aliquots at -20°C .

Cell culture system

LNCaP cells were obtained from the American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium-F12 medium, containing 10% fetal bovine serum, 1% L-glutamine, and 1% penicillin-streptomycin (complete medium [CM]), as described previously (18). Cells were typically cultured in complete medium before the culture conditions were altered. Androgen deprivation was achieved by culturing the cells in medium containing 10% charcoal-stripped serum (AD medium). Androgen was replaced by adding the synthetic androgen R1881 (NEN Life Science Products, Boston, MA) at 1×10^{-10} M to AD medium (18).

Western blot analyses

Protein levels of MDM2, p53, p21, Bcl-2, Bax, E2F1, pRb, and β -actin were analyzed after different treatments. Cells were cultured in complete, AD, or AD+R1881 medium for 3 days and incubated with 200 nM of AS or ASM in 4 mL culture medium for 24 h in the presence of 7 $\mu\text{g}/\text{mL}$ lipofectin (Invitrogen, Carlsbad, CA). Three hours after γ -irradiation to 5 Gy (RT) using a ^{137}Cs irradiator (Model 81-14R, J.L. Shepherd & Associates, San Fernando, CA), cells were lysed in a lysis buffer (50 mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate [SDS] with protease inhibitor cocktail set I [Calbiochem, San Diego, CA]) and were sonicated for 30 s on ice. Protein concentration was determined using the BCA protein assay reagent kit (Pierce, Rockford, IL). Identical amounts of protein were fractionated by SDS-PAGE electrophoresis and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). The membranes were then incubated in blocking buffer (phosphate-buffered saline containing 0.1% Tween 20 and

5% nonfat milk) for 1 h at room temperature and were washed twice with the washing buffer (phosphate-buffered saline containing 0.1% Tween 20) for 5 min. The membranes were then incubated with the appropriate primary antibody: anti-MDM2 monoclonal antibody (mAb) at 1:1000; anti-p53 mAb at 1:1000; anti-p21 mAb at 1:1000; anti-Rb mAb at 1:1000, anti- β actin at 1:5000 dilution or anti-E2F1 mAb at 1:1000 dilution (all antibodies from Calbiochem, San Diego, CA), anti-Bcl-2 mAb at 1:1000 (DAKO A/S, Carpinteria, CA), or anti-Bax polyclonal IgG at 1:1000 dilution (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) overnight at 4°C . Membranes were washed and then incubated with 1:2000 diluted sheep anti-mouse IgG or donkey-rabbit IgG horseradish peroxidase-conjugated secondary antibody (Amersham Pharmacia Biotech, Piscataway, NJ) for 1 h at room temperature. After the washes were repeated, the proteins of interest were detected by the enhanced chemiluminescence reagents according to the manufacturer's directions (Amersham, Aylesbury, UK).

Trypan blue cell viability assay

Early overall cell viability was assessed by trypan blue dye exclusion. Cells were seeded at 5×10^4 cells/well in 24-well plates and cultured in complete, AD, or AD+R1881 medium for 2–3 days. Cells were then transfected with 200 nM of AS or ASM in the presence of lipofectin (7 $\mu\text{g}/\text{mL}$). After 24 h, cells were irradiated to 5 Gy. The percentage of dead cells was measured by trypan blue dye exclusion at 24 and 48 h after treatment; typical cumulative cell death rates after AS treatment were 37% and 52%. From these data, the 48-h time point was chosen to be representative.

Measurements of apoptosis

Apoptosis was confirmed by Annexin V staining and Caspase 3+7 activity assays. LNCaP cells (2×10^5) were cultured in complete, AD, or AD+R1881 medium for 2–3 days. Cells were then incubated with 200 nM AS or ASM in the presence of lipofectin (7 $\mu\text{g}/\text{mL}$) for 18 h. Cells were then irradiated to 5 Gy. After 24 h, all cells (floating and attached) were harvested by trypsinization and labeled with Annexin V-PE and 7-amino-actinomycin D (7-AAD) (Guava Technologies Inc., Burlingame, CA) according to the manufacturer's instructions and analyzed by flow cytometry on a GuavaPC personal flow cytometer (Guava Technologies Inc., Burlingame, CA).

Caspase 3+7 activity was measured using a fluorometric substrate, Z-DEVD-Rhodamine (The Apo-ONE Homogeneous Caspase-3/7 Assay kit; Promega, Madison, WI). Cells were cultured for 2–3 days in CM, AD medium, or AD+R1881 medium and then incubated with AS or ASM for 18 h. Different times for AS exposure and the delay in performing the assay after RT were tested, and 18-h times were found to be representative, without excessive activity. Cells were then irradiated to 5 Gy. After 18 h, a total of 5×10^4 cells in 100 μL culture medium were mixed with 100 μL of Homogeneous Caspase-3/7 reagent in 96-well plates and incubated at room temperature for 18 h. Substrate cleavage was

Table 1. Western blot analyses of the effects of AD and/or RT on densitometry measurements of the expression of key proteins in the apoptotic pathway

| Group | n* | CM+RT÷ CM | AD+RT÷ AD | AD+R1881+RT÷ AD+R1881 |
|-------|----|--------------|--------------|--------------------------|
| p53 | 5 | 6.8 ± 2.0 | 2.8 ± 0.6 | 4.5 ± 1.6 |
| p21 | 3 | 3.0 ± 0.3 | 9.5 ± 2.5 | 7.7 ± 1.1 |
| Bcl-2 | 5 | 0.9 ± 0.1 | 1.1 ± 0.1 | 0.6 ± 0.1 |
| Bax | 3 | 0.5 ± 0.2 | 0.7 ± 0.1 | 1.1 ± 0.1 |
| MDM2 | 4 | 11.8 ± 3.3 | 26.8 ± 7.5 | 13.9 ± 5.2 |
| E2F1 | 3 | 1.1 ± 0.2 | 1.9 ± 0.3 | 1.9 ± 0.8 |
| pRb | 2 | 0.9 ± 0.1 | 0.9 ± 0.2 | 0.8 ± 0.3 |

* n = number of Western blot analyses done.

Note: The relative changes in band density measured by densitometry are shown as mean ± SEM.

quantified fluorometrically at 485-nm excitation and 538-nm emission. Fluorescence was measured on a fluorescent plate reader (LabSystems Inc., Franklin, MA). For a control, caspase 3+7 activity was inhibited by adding Ac-DEVD-CHO (Pro-mega, Madison, WI) to the cell culture before the assay.

Radiation treatment and clonogenic assay

Cells were cultured in complete, AD, or AD+R1881 medium for 2–3 days and then incubated with 200 nM AS or ASM in the presence of lipofectin (7 µg/mL). After 24 h, cells were irradiated to 2, 4, and 6 Gy. Immediately after irradiation, cells were trypsinized and serially diluted, and known numbers of cells were replated into 100-mm dishes. The plates were incubated for 12–14 days and stained with 0.25% methylene blue. The colonies were counted using an automated counter (Imaging Products International, Inc., Chantilly, VA). The clonogenic survival results were corrected for differences in plating efficiency from the various culture conditions. The dilutions for clonogenic assay were done in triplicate, and the results were averaged together (intraexperimental averages). The data shown in the clonogenic survival table represent the average from multiple experiments (interexperimental average).

RESULTS

Western blot analyses

MDM2 was identified as a potential target to enhance the response of prostate cancer cells to AD and RT through an investigation of the changes induced by these conditions in the expression of a variety of proteins involved in the apoptotic pathway. Table 1 displays the changes of MDM2, p53, p21, bcl-2, bax, E2F1, and pRb protein levels to AD ± RT, as determined by densitometry measurements of the resultant bands from Western blot analyses. The ratios of the band densities are shown. The average of 4 experiments of MDM2 revealed an 11.8-fold and a 26.8-fold increase in expression of MDM2 for CM+RT over CM and AD+RT over AD alone, respectively. When R1881 was added, the ratio of AD+RT over AD alone fell back to nearly the level

of the CM+RT over CM ratio. The changing level of MDM2 in response AD and RT was reflective of the changes in apoptosis under these conditions (18, 19). For these reasons, combined with the findings that p53 influences the apoptotic response of prostate epithelial cells to AD (8, 9), MDM2 was targeted using an antisense strategy.

Figure 1 displays representative Western blots showing that AS almost completely abrogated radiation-induced MDM2 expression in either complete, AD, or AD+R1881 medium, whereas ASM had little effect. The level of p53 increased after AS or RT treatment; ASM also increased the level of p53, as well as p21, but to a lesser degree. The mechanism for the slight increase in p53 levels after exposure to ASM is unclear, although in other Western blots, MDM2 seemed to be elevated from ASM treatment. The level of p21 was not increased by RT treatment, but was increased by AS treatment. AD alone had little effect on the protein levels of MDM2, p53, or p21. The expression of MDM2 seemed to be slightly higher for AS+AD+RT as compared to AS+AD, AS+RT, and AS+AD+R1881+RT. There was no obvious change in bcl-2 or bax expression by Western blot analysis in response to AS, AD, or RT (not shown).

Early cell death after AS ± AD ± RT treatment

The ability of AS to enhance the response of LNCaP cells to AD and/or RT was first evaluated using trypan blue dye exclusion. The cells were exposed to 200 nM AS, with or without AD, for 24 h, followed by γ-irradiation (5 Gy). A summary of three experiments measuring cell death 48 h after radiation is shown in Table 2. Eighteen treatment groups were analyzed together using analysis of variance. The statistics for the group comparisons are shown relative to the group above. Additional comparisons showed that AS resulted in significantly less cell death than AS+AD or AS+RT; these latter groups had about the same level of cell death. When R1881 was added to AS+AD, there was a reduction in cell death back to the level of AS. When AS was added to AD+RT, cell death was enhanced over all of the other groups, but the differences beyond that seen with AS+AD and AS+RT were not significant.

Direct measurements of apoptosis were performed to determine the contribution of apoptosis to early overall cell death that was quantified above by trypan blue staining. Apoptosis was measured directly by Annexin V binding. Cells were cultured in either complete, AD, or AD+R1881 medium for 48 h and then incubated with 200 nM AS or ASM for 18 h, followed by γ-irradiation (5 Gy). Twenty-four hours after irradiation, cells were prepared for Annexin V-PE and 7-AAD staining. Table 3 shows that early apoptosis (Annexin V-PE-positive and 7-AAD-negative) was higher from AS+AD (36.6% apoptosis) and AS+RT (32.7%) treatments over either AS (22.2%), AD (6.7%), or RT (3.9%) treatments given individually. These findings were significant (Table 2). However, there was no significant difference between AS+AD or AS+RT and AD+AS+RT, although the level of apoptosis was consistently higher in the AD+AS+RT group.

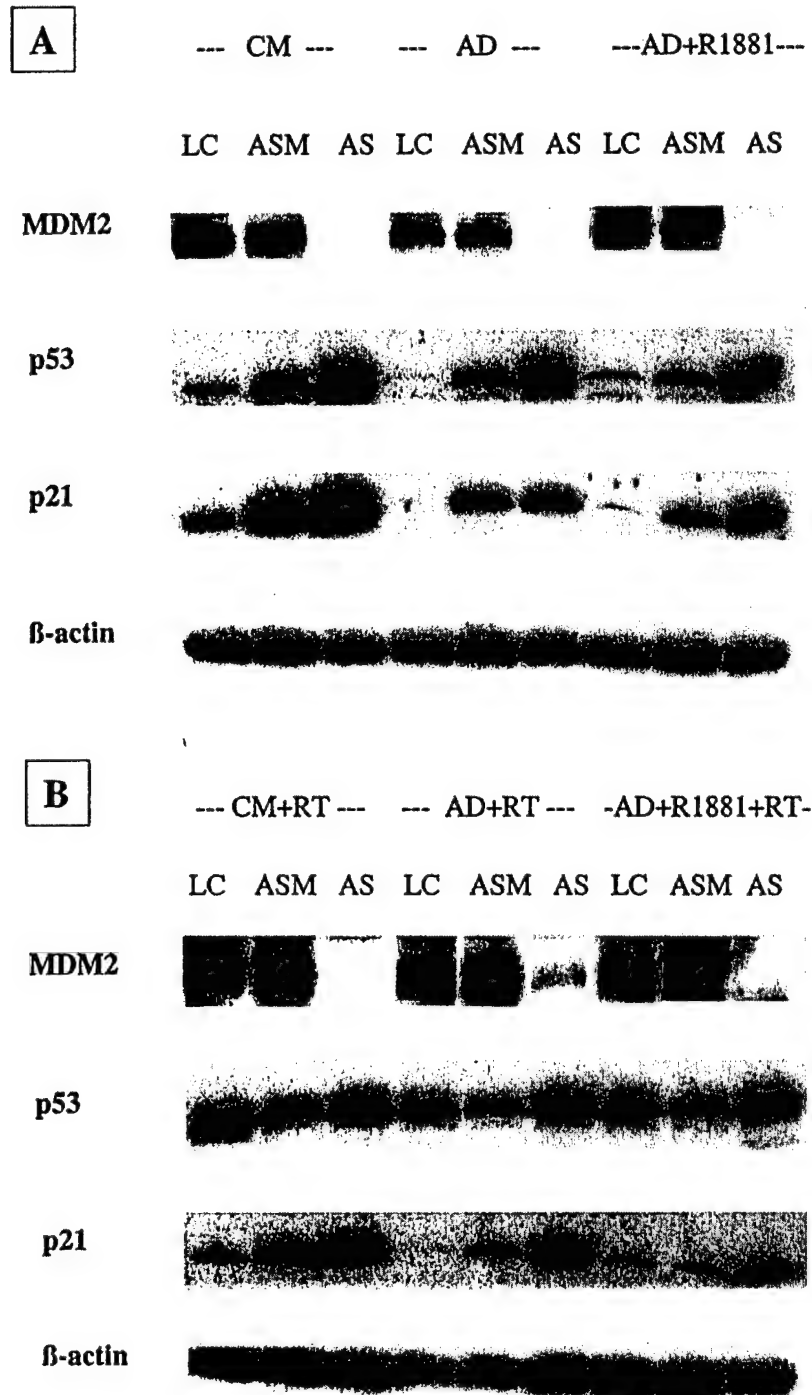


Fig. 1. Western blot analyses of LNCaP cells grown for 2–3 days in CM, AD, or AD+R1881 medium. AS or ASM was administered at 200 nM; 24 h later, RT at 5 Gy was given. The cells were harvested 3 h later, and the protein was extracted for analysis of MDM2, p53, p21, and β -actin levels. (A) Without RT; (B) With RT.

The pattern of apoptotic cell death observed by the Annexin V assay was very similar to that from the Caspase 3+7 assay. As shown in Table 4, Caspase 3+7 activity was increased from AS+AD or AS+RT as compared to AS, AD or RT treatments given singly. There was no significant increase in apoptosis from AS+AD+RT over that from AS+AD or AS+RT.

Caspase 3+7 activity was inhibited by the addition of R1881 to AS+AD to approximately the levels of AS alone. Moreover, the addition of specific caspase inhibitor Ac-DEVD-CHO (data not shown) reduced caspase 3+7 activity. These results suggest that AS accentuates LNCaP tumor cell apoptosis to AD and RT through p53 by activating caspase 3+7.

Table 2. Trypan blue quantification of early cell death

| Treatment | Mean | SEM | p* |
|-----------------------------|------|-----|---------|
| Lipofectin control | 10.8 | 0.5 | — |
| AS [†] | 52.0 | 3.4 | <0.0001 |
| ASM | 24.8 | 1.4 | <0.0001 |
| AD | 21.3 | 1.4 | 1.000 |
| AD+AS ^{†,‡} | 71.0 | 3.9 | <0.0001 |
| AD+ASM | 31.8 | 1.9 | <0.0001 |
| AD+R1881 | 15.5 | 2.1 | 0.226 |
| AD+AS+R1881 [†] | 57.0 | 5.6 | <0.0001 |
| AD+ASM+R1881 | 30.0 | 3.9 | <0.0001 |
| Lipofectin control+RT | 21.8 | 2.2 | 1.000 |
| AS+RT ^{†§} | 69.5 | 3.4 | <0.0001 |
| ASM+RT | 30.8 | 3.7 | <0.0001 |
| AD+RT [†] | 26.3 | 2.2 | 1.000 |
| AD+AS+RT [§] | 82.3 | 3.5 | <0.0001 |
| AD+ASM+RT | 39.3 | 4.2 | <0.0001 |
| AD+R1881+RT | 19.3 | 1.1 | 0.02 |
| AD+R1881+AS+RT [§] | 69.0 | 4.1 | <0.0001 |
| AD+R1881+ASM+RT | 35.8 | 6.7 | <0.0001 |

Abbreviations: AS = antisense MDM2; ASM = antisense mismatch; AD = androgen deprivation; RT = radiation therapy; SEM = standard error of the mean.

* Compared to group above, one-way ANOVA, Bonferroni test. The average of 4 experiments is shown.

[†] AD+AS vs. AS ($p = 0.039$); AD+AS vs. AD+AS+R1881 ($p = 0.855$).

[‡] AD+AS vs. AS+RT ($p = 1.000$); AD+AS vs. AD+RT ($p < 0.0001$).

[§] AD+AS vs. AD+AS+RT ($p = 1.000$); AS+RT vs. AD+AS+RT ($p = 1.000$); AD+AS+RT vs. AD+R1881+AS+RT ($p = 1.000$).

Overall cell death by clonogenic cell survival assay

Clonogenic cell survival experiments were performed to determine whether the added, but not significant, early cell killing from apoptosis due to AS+AD+RT translates into a significant increase in overall cell killing, i.e., the cell killing manifested over time. The early cell death measurements by trypan blue and the apoptosis markers may not be representative of all cell death occurring over time. Figure 2 shows the clonogenic assay results for LNCaP cells grown for 2–3 days in CM and then treated with lipofectin alone, AS, or ASM for 24 h before RT. The cells were then replated immediately after RT at 2, 4, or 6 Gy. The results show LNCaP radiosensitization by AS at all RT dose levels, over the CM and ASM controls. Figure 3 reveals that radiosensitization was further enhanced when AD was added to AS and that this effect was reduced by R1881 supplementation. The radiosensitizing action of AS+AD was much greater than the minor effect observed from ASM+AD.

DISCUSSION

Androgen deprivation and RT are central to the treatment of prostate cancer patients with high-risk prostate cancer. Even with the gains seen from this combination over single-modality therapy, the outcome of such high-

Table 3. Annexin V quantification of early apoptosis

| Treatment | Mean | SEM | p* |
|-----------------------------|------|-----|---------|
| Lipofectin control | 3.4 | 0.7 | — |
| AS [†] | 22.2 | 0.5 | <0.0001 |
| ASM | 7.4 | 0.4 | <0.0001 |
| AD | 6.7 | 0.8 | 1.000 |
| AD+AS ^{†,‡} | 36.6 | 1.2 | <0.0001 |
| AD+ASM | 12.1 | 1.1 | <0.0001 |
| AD+R1881 | 5.7 | 1.1 | 0.225 |
| AD+AS+R1881 [†] | 28.6 | 2.0 | <0.0001 |
| AD+ASM+R1881 | 10.7 | 1.4 | <0.0001 |
| Lipofectin control+RT | 3.9 | 0.8 | 0.219 |
| AS+RT ^{†§} | 32.7 | 1.4 | <0.0001 |
| ASM+RT | 9.6 | 1.6 | <0.0001 |
| AD+RT [†] | 7.2 | 1.0 | 1.000 |
| AD+AS+RT [§] | 40.4 | 2.5 | <0.0001 |
| AD+ASM+RT | 16.2 | 3.0 | <0.0001 |
| AD+R1881+RT | 5.3 | 1.1 | 0.0001 |
| AD+R1881+AS+RT [§] | 34.3 | 1.8 | <0.0001 |
| AD+R1881+ASM+RT | 12.2 | 2.1 | <0.0001 |

Abbreviations: AS = antisense MDM2; ASM = antisense mismatch; AD = androgen deprivation; RT = radiation therapy; SEM = standard error of the mean.

* Compared to group above, one-way ANOVA, Bonferroni test. The average of 4 experiments is shown.

[†] AD+AS vs. AS ($p < 0.0001$); AD+AS vs. AD+AS+R1881 ($p = 0.018$).

[‡] AD+AS vs. AS+RT ($p = 1.000$); AD+AS vs. AD+RT ($p < 0.0001$).

[§] AD+AS vs. AD+AS+RT ($p = 0.106$); AS+RT vs. AD+AS+RT ($p < 0.0001$); AD+AS+RT vs. AD+R1881+AS+RT ($p = 0.964$).

risk patients remains rather poor. An understanding of the mechanisms of the interaction between AD and RT could lead to novel therapies that dramatically alter the failure profile.

Prior studies have indicated that p53 may have a role in the apoptotic response of prostate epithelial cells to AD (20). The results, however, have not been conclusive (21, 22). Little is known about why most prostate cancers respond to AD preferentially with a shift from cell proliferation to quiescence in the setting of minimal increases in apoptosis (23–28). There must be a key regulatory defect in the apoptotic pathway that preferentially shunts cells into quiescence instead of apoptosis. The data presented here point to MDM2. Of all of the proteins in the apoptotic pathway examined, MDM2 expression levels fluctuated in tandem with previously defined changes in apoptosis in response to AD+RT. We recently reported that in LNCaP cells grown *in vitro* (18) and in R3327-G Dunning rat prostate tumors grown *in vivo* (19), when AD precedes RT by 3 days, a supra-additive apoptotic response, over AD or RT given individually, is evidenced. Although supra-additive apoptosis was observed, the extent of the supra-additive response was rather minimal. The general lack of apoptosis seen in the response of prostate cancer cells to AD or RT alone, and

Table 4. Caspase 3+7 quantification of early apoptosis

| Treatment | Mean | SEM | <i>p</i> * |
|-----------------------|------|-----|------------|
| Lipofectin control | 114 | 16 | — |
| AS† | 335 | 19 | <0.0001 |
| ASM | 199 | 25 | 0.169 |
| AD | 73 | 20 | 0.333 |
| AD+AS†‡ | 504 | 7 | <0.0001 |
| AD+ASM | 215 | 13 | <0.0001 |
| AD+R1881 | 109 | 29 | 1.000 |
| AD+AS+R1881† | 349 | 20 | <0.0001 |
| AD+ASM+R1881 | 170 | 33 | 0.006 |
| Lipofectin control+RT | 89 | 10 | 1.000 |
| AS+RT‡§ | 547 | 46 | <0.0001 |
| ASM+RT | 259 | 27 | <0.0001 |
| AD+RT‡ | 112 | 15 | 0.071 |
| AD+AS+RT§ | 610 | 35 | <0.0001 |
| AD+ASM+RT | 302 | 15 | <0.0001 |
| AD+R1881+RT | 90 | 21 | <0.0001 |
| AD+R1881+AS+RT§ | 491 | 50 | <0.0001 |
| AD+R1881+ASM+RT | 218 | 38 | <0.0001 |

Abbreviations: AS = antisense MDM2; ASM = antisense mismatch; AD = androgen deprivation; RT = radiation therapy; SEM = standard error of the mean.

* Compared to group above, one-way ANOVA, Bonferroni test. The average of 3 experiments is shown.

† AD+AS vs. AS ($p = 0.014$); AD+AS vs. AD+AS+R1881 ($p = 0.039$).

‡ AD+AS vs. AS+RT ($p = 1.000$); AD+AS vs. AD+RT ($p < 0.0001$).

§ AD+AS vs. AD+AS+RT ($p = 1.000$); AS+RT vs. AD+AS+RT ($p = 1.000$); AD+AS+RT vs. AD+R1881+AS+RT ($p = 0.571$).

the modest short-lived increase in apoptosis from the combination, suggest that apoptosis is being suppressed.

Under the conditions of AD+RT, the relative levels of MDM2 increase, as compared to AD alone or AD+RT+R1881. In light of the increase in apoptosis levels observed herein when MDM2 expression is suppressed, it seems that the increase in MDM2 in response to AD+RT is due to feedback regulation, such that MDM2 dampens what would otherwise be a very pronounced apoptotic response in normal prostate epithelial cells. Because overexpression of MDM2 is seen in 30%–40% of prostate cancers, the action of MDM2 on response to AD and/or RT has significant clinical implications.

Previously we found that the suppression of MDM2, through the use of antisense MDM2 oligonucleotides (AS), not only induces significant levels of apoptosis in LNCaP cells by itself, but also results in a pronounced enhancement in apoptosis when combined with AD (17). Those findings have been substantiated and extended in this communication. The main question posed here was whether AS sensitizes cells to the combination of AD+RT when all of the other possible treatments are considered. Antisense MDM2 has been shown to sensitize tumor cells to radiation (13). Radiosensitization in terms of the apoptotic response by AS was confirmed in LNCaP cells. Both AD+AS and RT+AS displayed greater levels of apoptosis than the sum of the individual treatments. When all three treatments were combined, there was a consistent, albeit insignificant, increase in apoptosis seen over AD+AS or RT+AS. Because apoptosis was measured at a single point in time and may not be reflective of overall cell killing, clonogenic cell survival assays were performed.

By clonogenic assay, AS has been shown previously to

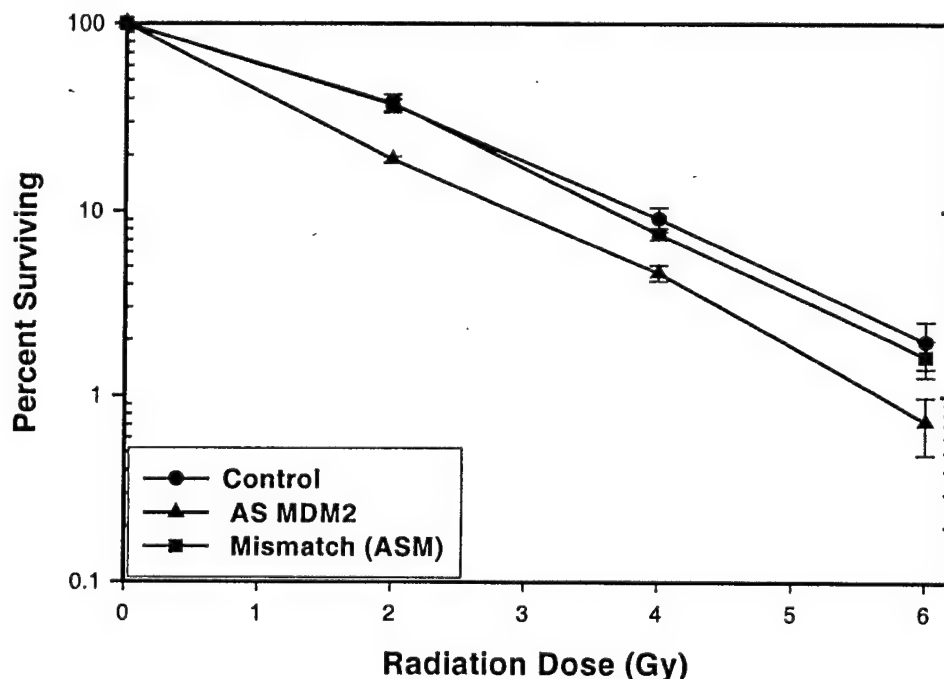


Fig. 2. Clonogenic assays of LNCaP cells cultured in CM alone or with AS or ASM (200 nM) added for 24 h before RT at 2, 4, or 6 Gy.

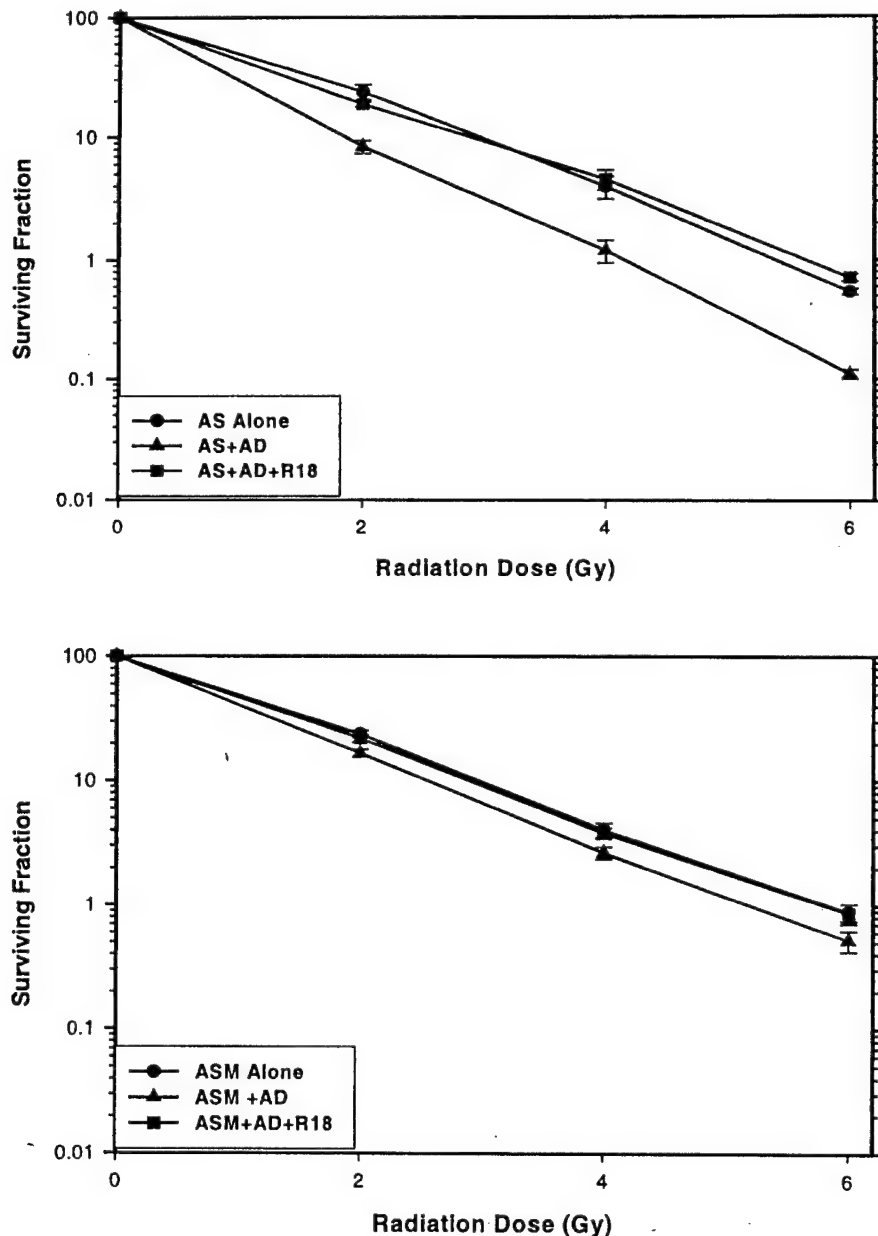


Fig. 3. Clonogenic assays of LNCaP cells cultured in CM or AD medium for 48–72 h and exposed to AS or ASM (200 nM) for 24 h before RT at 2, 4, or 6 Gy. AS = antisense MDM2; AD = androgen deprivation; R18 = synthetic androgen R1881; ASM = antisense mismatch.

significantly reduce clonogen survival when added to AD, as compared to each treatment applied individually. We show here that AS is also a potent radiosensitizer. Moreover, a further reduction of clonogen survival was evidenced when AS+AD+RT were given together, as compared to the controls (Fig. 3). The reduction in clonogenic cell survival was significant, and seemed to be greater than that observed by apoptosis alone. This could be related to the technical difficulty in summing apoptosis over time, which we did not attempt to do, or to other effects on cell survival, such as mitotic cell death. In either case, the data

substantiate the critical role of MDM2 in the response of prostate cancer cells to AD and RT.

CONCLUSION

In summary, MDM2 is emerging as a central regulatory component in the cell death response of prostate cancer cells to AD and RT and has the potential to be manipulated therapeutically with AS. The hypothesized

mechanism for AS action is alteration of p53 expression via effects on MDM2 (the LNCaP cell line used is wild type for p53), although p53-independent effects may also contribute (7, 29). By enhancing the cell death response to AD, AS should improve cure rates by promoting cell

death in micrometastatic deposits, as well as reduce the number of clonogens at the primary site. The reduction in clonogens from AD+AS, when combined with the radiosensitizing effects of AS, makes this strategy ideal for the man with high-risk prostate cancer.

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Antisense MDM2 Oligonucleotides Restore the Apoptotic Response of Prostate Cancer Cells to Androgen Deprivation

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BACKGROUND. Early in the malignant transformation of prostate epithelial cells, the apoptotic response to androgen deprivation (AD) is lost and the principle response is a slowing of cell growth. In this study, we tested whether interruption of MDM2 function using antisense MDM2 oligonucleotide (AS) affects the apoptotic response of prostate cancer cells to AD.

METHODS. Wild type LNCaP cells and MDM2-overexpressing (LNCaP-MST) cells were treated with AS alone or in combination with AD. Protein levels of MDM2, p53, and p21 were determined by Western blotting. Cell viability was measure by trypan blue staining. Apoptotic cell death was confirmed by cell morphological changes, annexin V/propidium iodide staining and caspase-3 + 7 activity. Overall cell survival was quantified by clonogenic assay.

RESULTS. AS inhibited MDM2 expression to a greater extent in LNCaP cells, as compared to LNCaP-MST cells. AS enhanced the expression of p53 and p21 in both cell lines. The growth inhibitory and cell death effects of AS + AD were generally greater than AS alone in LNCaP cells. Treatment of LNCaP cells with AS + AD for 72 hr caused a significant increase in cell death (66%) over AD alone (13%), AS alone (33%), or AD + AS + R1881 (34% with synthetic androgen replacement) that was attributable mainly to apoptosis. Clonogenic survival reflected the same pattern.

CONCLUSIONS. Our results suggest that the apoptotic response of prostate cancer to AD is strongly influenced by MDM2 expression. Antisense MDM2 has broad potential as a therapeutic agent to sensitize prostate cancer cells to AD therapy by enhancing apoptotic cell death. *Prostate* 9999: 1-10, 2004. © 2004 Wiley-Liss, Inc.

KEY WORDS: apoptosis; annexin V; caspase; clonogenic survival

INTRODUCTION

Androgen deprivation (AD) is the most common treatment for advanced prostate cancer. Although this therapy successfully results in dramatic regression of prostate tumors, the responses are generally

temporary, and eventually progression to androgen independence occurs in the vast majority of cases [1]. AD is effective at causing tumor shrinkage by promoting apoptosis and a shift to quiescence [2,3]. Although normal prostate epithelial cells exhibit an extensive

Abbreviations: AD, androgen deprivation; AS, anti-MDM2 antisense; RT, radiation therapy; CM, completed medium; AD medium, charcoal stripped serum containing medium; R1881, synthetic androgen; LNCaP, human prostate cancer cell line that has wild-type expression of MDM2; LNCaP-MST, stably-transfected LNCaP cells which overexpress MDM2. [The contents are solely the responsibility of the authors and do not necessarily represent the official views of the National Cancer Institute or the US Department of Defense^{Q2}.]

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apoptotic response to AD [4], it appears that this response is appreciably reduced or lost early in malignant transformation [5–9]. The main effect of AD in these cases is a reduction in cell proliferation. If the apoptotic response of prostate cancer to AD could be restored through manipulation of the intracellular molecular milieu, it may be possible to enhance and prolong clinical responses, and perhaps ultimately patient survival.

MDM2 is an oncogene and the gene product is a key protein in the apoptotic pathway which binds to p53, E2F-1, and pRb, as well as other proteins [10]. It is induced by p53, binds to p53 with high affinity, and is a pivotal negative regulator of p53 action. The *MDM2* gene is amplified in a variety of human tumors [11], including prostate cancer [12–15]. The expression of MDM2 is increased in prostate cancer patients with high risk local-regional disease [16] and with the development of hormone refractory disease [9]. MDM2 is also a target for cancer therapy. Suppression of MDM2 using anti-MDM2 antisense (AS) sensitizes tumor cells to radiation [17] and chemotherapy [18–21].

Little is known about the molecular mechanisms that govern prostate cancer response to AD. Although functional p53 protein is not essential for the apoptotic response of normal prostate epithelial cells to castration [22,23], abnormal or suppressed p53 expression has been associated with resistance to AD [24–26]. Since MDM2 ablates p53 function, we hypothesized that MDM2 may contribute to the modulation of the apoptotic response to AD. In this study, the role of MDM2 was examined by suppressing MDM2 expression with a second generation antisense oligonucleotide (AS). The effects of AS were tested on wild type LNCaP and MDM2-overexpressing (LNCaP-MST) cells.

MATERIALS AND METHODS

Antisense Oligonucleotides

The antisense MDM2 20-mer mixed-backbone oligonucleotide (AS; 5'-UGACACCTGTTCTCACUCAC-3') and its mismatch control oligonucleotide (ASM; 5'-UGTCACCTTTTTTCATUCAC-3') were obtained from Hybridon, Inc. (Cambridge, MA). They were stored as frozen aliquots at -20°C .

Cell Culture and Transfection

LNCaP cells were cultured in Dulbecco's modified Eagle's medium (DMEM)-F12 medium, containing 10% fetal bovine serum (FBS), 1% L-glutamine, and 1% penicillin-streptomycin (complete medium, CM), as described previously [27]. Cells were typically cultured for 24 hr in CM before the culture conditions

were altered. AD was achieved by culture in 10% charcoal-stripped serum containing medium (AD medium). Replacement of androgen was done by adding the synthetic androgen R1881 (NEN Life Science Products, Boston, MA) at 1×10^{-10} M to AD medium [27].

To establish stable transfectants of MDM2 in LNCaP cells, cells were seeded at 1×10^6 cells per 10 cm dish for 24 hr and transfected with the pCMV-mdm2 expression plasmid by lipofectamine according to the manufacturer's procedure (Invitrogen, Carlsbad, CA). Neomycin-resistant cells were selected in the presence of 800 $\mu\text{g}/\text{ml}$ Geneticin (G418, Life Technologies, Gaithersburg, MD) 48 hr after transfection. Expression of MDM2 was tested by Western blot analysis of cells obtained from a single colony.

Western Blot Analysis

Cells (1×10^6 /dish) were incubated with 200 nM of AS or ASM in 4 ml culture medium for 24 hr in the presence of 7 $\mu\text{g}/\text{ml}$ lipofectin (Invitrogen). Cells were lysed in a lysis buffer (50 mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate (SDS) with protease inhibitor cocktail set I (Calbiochem, San Diego, CA) and were sonicated. Protein concentration was determined using the BCA protein assay reagent kit (Pierce, Rockford, IL). Equal amounts of protein (50 μg) were separated by SDS-PAGE. After electrophoresis, proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA). The membranes were incubated with blocking buffer (PBS containing 0.1% Tween-20 and 5% non-fat milk) for 1 hr at room temperature and were washed twice in washing buffer (PBS containing 0.1% Tween-20) for 5 min. Membranes were immunoprobed with either anti-MDM2 monoclonal antibody (mAb) at 1:1,000, anti-p53 mAb at 1:1,000, anti-p21 mAb at 1:1,000, or anti- β actin at 1:5,000 dilution (Calbiochem), incubating overnight at 4°C . Membranes were then washed and incubated with 1:2,000 diluted sheep antimouse IgG horse-radish peroxidase conjugated secondary antibody (Amersham Life Science) for 1 hr at room temperature. After washing, the proteins were detected by the enhanced chemiluminescence reagents according to the manufacturer's direction (Amersham, Aylesbury, UK).

Cell Growth and Cell Viability

For the cell growth experiments, cells were plated in 24-well plates at 2.5×10^4 cells/well and cultured in CM for 24 hr. The medium was then changed and replaced with either CM, AD medium, or AD + R1881 medium. Simultaneous with the medium change, 20 nM of AS or ASM was added into medium in the

presence of lipofectin (7 μ g/ml). The number of viable cells was counted at 24 hr intervals.

For the cell viability assay, 5×10^4 cells/well were seeded in 24-well plates. Cells were treated with 200 nM of AS or ASM and the percentage of dead cells measured by trypan blue dye exclusion at various times.

Confirmation of Apoptosis

24-48 Two methods were used to confirm apoptotic cell death. LNCaP cells were cultured in either CM, AD, medium, or AD + R1881 medium for 48 hr. Cells were then incubated with 200 nM AS or ASM in 4 ml of medium for 48 hr in the presence of lipofectin (7 μ g/ml). Cell morphological changes were examined by light microscopy.

Annexin V staining was used to determine the proportion of apoptotic cells. After incubation with 200 nM AS or ASM, all cells (floating and attached) were harvested by trypsinization, labeled with annexin V-PE and 7-amino-actinomycin D (7-AAD) (Guava Technologies, Inc., Burlingame, CA) according to the manufacturer's instructions and analyzed by flow cytometry on a GuavaPC personal flow cytometer.

Caspase-3 + 7 activity was measured using a fluorometric substrate, Z-DEVD-Rhodamine (The Ap α -ONE™ Homogeneous Caspase-3/7 Assay kit; Promega, Madison, WI). Cells were cultured in CM, AD, or AD + R1881 medium for 2 days and then incubated with AS or ASM (200 nM) for 30 hr. A total of 5×10^4 cells in 100 μ l culture medium were mixed with 100 μ l of Homogeneous Caspase-3/7 reagent in 96-well plates and incubated at room temperature for 18 hr. Substrate cleavage was quantified fluorometrically at 485 excitation and 538 nm emission. Fluorescence was measured on a fluorescent plate reader (LabSystems, Inc., Franklin, MA). As a control, caspase-3 + 7 activity was inhibited in some cells by adding Ac-DEVD-CHO (Promega) to the culture before the assay.

Clonogenic Assay

Clonogenic assays were performed as described previously [28]. In brief, cells were cultured in CM, AD medium, or AD + R1881 medium for 48 hr and then incubated with AS or ASM at various concentrations for 24 hr in presence of lipofectin (7 μ g/ml). The cells were trypsinized, serially diluted, and known numbers of cells replated into 100 mm dishes. The plates were incubated for 12–14 days and stained with 0.25% methylene blue. The number of colonies was determined using an automated counter (Imaging products International, Inc., Chantilly, VA). The clonogenic survival results were corrected for differences in plating efficiency from the various culture conditions. The dilutions for clonogenic assay were done in

triplicate and the results were averaged together (intra-experiment averages). The data shown in the clonogenic survival table represent an average from multiple experiments (inter-experiment averages).

RESULTS

overexpressed

Effect of AS on MDM2, p53, and p21 Protein Levels

To evaluate the effects of MDM2 suppression and overexpression on AD, stable transfectants of LNCaP cells that overexpress human MDM2 were established. As displayed in Figure 1, expression of MDM2 was increased substantially in LNCaP-MST cells, compared to parental LNCaP cells.

The effects of AS on MDM2, p53, and p21 expression in both LNCaP and LNCaP-MST cells were measured. Figure 1 shows that AS decreased the expression of MDM2 and increased the expression of both p53 and p21 in a dose-dependent manner in LNCaP and LNCaP-MST cells. There appears to be less of a reduction of MDM2 expression with high concentrations of AS (500 nM) in LNCaP-MST cells; although, this could be related to preferential cell death and loss of the subpopulation of cells with low MDM2 levels. Treatment with ASM appeared to have a slight stimulatory effect on MDM2 expression and little effect on p53 and p21 protein levels, even at 500 nM.

Studies were then performed to determine the action of AS on LNCaP and LNCaP-MST cells grown in vitro in Δ androgen deprived (AD) medium. After 2 days of culture in either CM, AD medium, or AD + R1881 medium, cells were incubated with 200 nM AS or ASM in the presence of lipofectin for 24 hr. As illustrated in Figure 1C, AD alone caused a reduction in MDM2, p53, and p21 protein levels in LNCaP cells. When AS was combined with AD, MDM2 expression in LNCaP cells was further reduced and the expression of p53 and p21 remained high. When R1881 was added to LNCaP cells treated with AD, there was little alteration in MDM2, p53, and p21 levels from the addition of AS or ASM, over that seen with AD alone. Figure 1D shows that AD caused slight reductions in MDM2 and p53 levels, and a larger reduction in p21 expression in LNCaP-MST cells. Treatment of LNCaP-MST cells with AS + AD further reduced MDM2 levels, while the expression of p53 and p21 remained high. When R1881 was added to LNCaP-MST cells treated with AD, there was little alteration in MDM2, p53 and p21 levels from the addition of AS or ASM, over that seen with AD alone.

LNCaP and LNCaP-MST Cell Growth and Viability After AS \pm AD Treatment

Previous studies have shown that the growth of LNCaP cells is inhibited considerably when cultured in

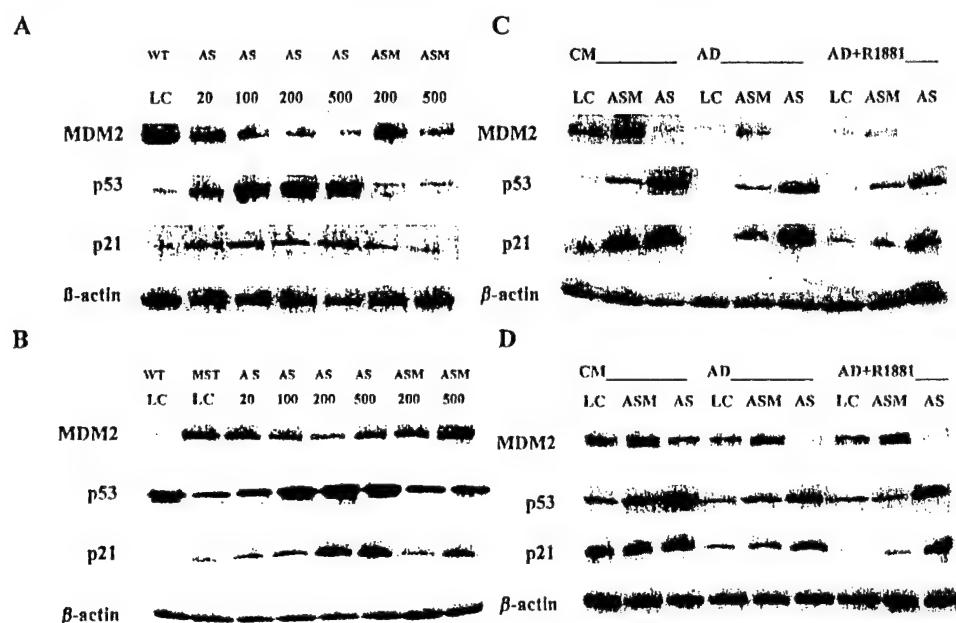


Fig. 1. Western blot analysis of MDM2, p53, and p21 levels in LNCaP (WT, panel A) and LNCaP-MST (MST, panel B) cells exposed to different concentrations of AS (in nM) for 24 hr. Western blot analysis of MDM2, p53, and p21 levels in cells grown in complete, AD, and AD + R1881 medium is shown in panel C for LNCaP and panel D for LNCaP-MST cells. Total protein was 30 μ g per lane. LC, lipofectin control; AD, androgen deprivation; AS, antisense MDM2.

AD medium and is partially reversed by adding the synthetic androgen R1881 back to AD medium [12]. To evaluate whether AS combined with AD resulted in enhanced LNCaP cell growth inhibition over AS or AD given individually, different doses of AS (10–200 nM) were first tested. Cell growth inhibition was dose-dependent (data not shown). LNCaP cells were then treated with 20 nM AS or ASM with or without AD for 4 days. The findings depicted in Figure 2A illustrate that the replication of LNCaP cells was completely

suppressed after exposure to AS + AD; the combination resulted in more growth inhibition than the individual treatments with AS or AD ($P < 0.001$, one way analysis of variance, Bonferroni test at day 3 of culture). The synthetic androgen R1881 partially restored the growth of LNCaP cells treated with AS + AD such that cell number at day 3 was higher with R1881 added, as compared to AS + AD alone ($P = 0.001$, Bonferroni test). The growth inhibitory effects of AS, AD, and AS + AD were less overall in

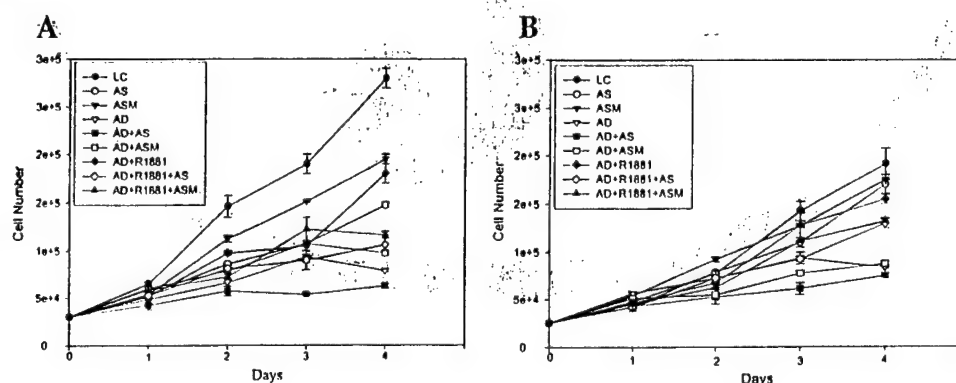


Fig. 2. LNCaP (A) and LNCaP-MST (B) cell growth inhibition in vitro by AS or ASM in combination with AD or AD + R1881. Cells were cultured in complete medium (CM) for 24 hr, then the medium was changed and the cells cultured further in CM, AD, or AD + R1881 medium. Simultaneously 20 nM AS or ASM was added to medium in presence of lipofectin. The number of viable cells were counted at the indicated times. LC, lipofectin control.

LNCaP-MST cells; although the general pattern for the individual and combined treatments was similar (Fig. 2B).

Cell Death From AS ± AD

The early overall cell death response of LNCaP cells to AS, AD and the combination was first examined using trypan blue dye uptake. The cells were exposed to 200 nM AS with or without AD for different periods of time. A summary of three experiments measuring LNCaP cell death after 72 hr is shown in Table I. Cell death from AS + AD was significantly enhanced (66%) over that of ASM + AD (22%), AS alone (33%), or AD alone (13%). The cell killing effect of AS + AD was significantly reversed to 34% when the synthetic androgen R1881 was added. Table II displays the cell death effects of AS, AD and the combination on LNCaP-MST cells. While there was a significant increase in cell death from AS + AD over AS or AD alone, and this effect was reversed by R1881, the extent of these differences was less than was seen in the LNCaP line. The proportions of cells dying after incubation of LNCaP and LNCaP-MST cells in lipofectin only or AD alone were comparable.

Figure 3 shows the early cell death response of LNCaP cells to AD alone, ASM + AD, and AS + AD in terms of the morphological effects, which are suggestive of apoptosis. These effects included decreased cell density, elongation and shrinkage of cells by 24 hr, rounding of some cells, and ~~lost anchorage~~ and detachment from the culture surface by 48 hr. These morphological changes were more pronounced from AS + AD than from AD alone or ASM + AD.

TABLE I. Effect of Antisense MDM2 on LNCaP Cell Death Measured by Trypan Blue Dye Uptake

| Treatment | Mean | SEM | P* |
|---------------------------|------|-----|---------|
| Lipofectin control | 5.8 | 0.7 | — |
| AS (Antisense MDM2) | 32.8 | 1.5 | <0.0001 |
| ASM (mismatch control) | 15.7 | 0.2 | <0.0001 |
| AD (androgen deprivation) | 13.5 | 0.3 | 1.000 |
| AD + AS** | 66.0 | 1.5 | <0.0001 |
| AD + ASM | 21.8 | 2.7 | <0.0001 |
| AD + R1881 | 7.5 | 1.3 | <0.0001 |
| AD + AS + R1881 | 34.0 | 0.8 | <0.0001 |
| AD + ASM + R1881 | 16.5 | 0.8 | <0.0001 |

Percentage of cell death measured by trypan blue dye uptake after 3 days of culture. The data shown represent the average values (±SEM) from three independent experiments.

*Compared to group above, one way Anova, Bonferroni test.

**Other comparison: AD + AS versus AD + AS + R1881 ($P < 0.0001$); AD + AS versus AS ($P < 0.0001$).

TABLE II. Effect of Antisense MDM2 on LNCaP-MST Cell Death Measured by Trypan Blue Dye Uptake

| Treatment | Mean | SEM | P* |
|---------------------------|------|-----|---------|
| Lipofectin control | 6.3 | 0.9 | — |
| AS (antisense MDM2) | 19.0 | 4.6 | 0.003 |
| ASM (mismatch control) | 12.3 | 0.9 | 0.604 |
| AD (androgen deprivation) | 12.3 | 1.5 | 1.000 |
| AD + AS** | 32.0 | 1.2 | <0.0001 |
| AD + ASM | 17.0 | 0.6 | <0.0001 |
| AD + R1881 | 10.0 | 1.2 | 0.456 |
| AD + AS + R1881 | 19.0 | 0.6 | 0.081 |
| AD + ASM + R1881 | 13.7 | 0.9 | 1.000 |

Percentage of cell death measured by trypan blue dye uptake after 3 days of culture. The data shown represent the average values (±SEM) from three independent experiments.

*Compared to group above, one way Anova, Bonferroni test.

**Other comparisons: AD + AS versus AD + AS + R1881 ($P = 0.002$); AD + AS versus AS ($P = 0.002$).

Direct measurements of apoptosis were performed to determine the contribution of apoptosis to early overall cell death from AS ± AD. Apoptosis was measured directly by annexin V binding. The cells were cultured in either complete, AD, or AD + R1881 medium for 48 hr and then incubated with AS or ASM for 48 hr. Table III displays the results of annexin V staining in LNCaP cells, revealing that the cells undergoing early apoptosis (annexin-V-PE-positive and 7-AAD-negative) increased to 26.5% after AS + AD AD treatment, relative to 17.5 and 8.5% for AS and AD given individually. The difference was significant for AS + AD versus AD alone, but not for AS + AD versus AS alone (Table III). Table IV shows that a similar trend was observed for the effect of AS on the apoptosis of LNCaP-MST cells; however, the addition of AD to AS did not result in an enhanced response over AS alone. The lack of sensitization of LNCaP-MST cells to AS + AD could be related to the greater amount of apoptosis seen with AS alone (Table IV), as compared to LNCaP cells (Table III). A similar pattern was apparent in the caspase-3 + 7 experiments (Tables V and VI).

Caspase-3 + 7 activity is another marker of apoptosis that was used to ~~substantiate~~ ^{corroborate} the annexin V findings. The caspase data reflected that obtained with annexin V staining. After AS exposure, caspase-3 + 7 activity was higher in LNCaP-MST cells (Table VI) than in LNCaP cells (Table V). In LNCaP cells, AS + AD resulted in significantly elevated caspase-3 + 7 levels, compared to AS and AD applied individually (Table V). Moreover, caspase-3 + 7 activity was inhibited by the addition of R1881 to AS + AD to approximately the levels of AS alone. The addition of specific caspase inhibitor Ac-DEVD-CHO (data not shown) reduced caspase-3 + 7

comparisons

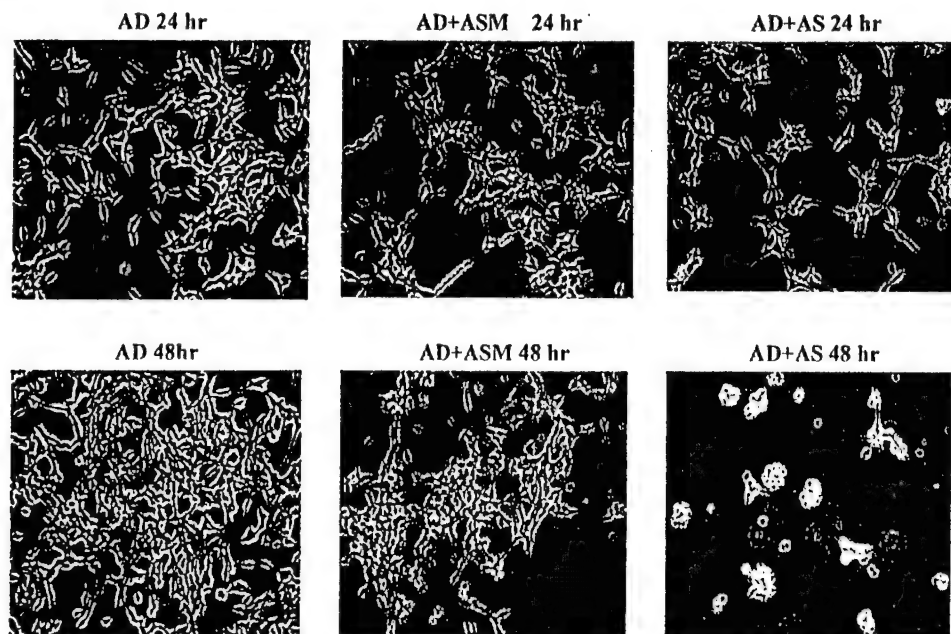


Fig. 3. Morphology of LNCaP cells after treatment for 24 and 48 hr with AS or ASM (200 nM), and AD or AD + R1881. The cells were examined under light microscopy magnification of 200 \times .

activity. In LNCaP-MST cells, AS caused a significant increase in apoptosis over ASM and the lipofectin control, but the addition of AD to AS did not result in a further increase in apoptosis (Table VI). These results suggest that AS restores LNCaP tumor cell apoptosis to AD through p53 by activating caspase-3 + 7, and that the overexpression of MDM2 inhibits or delays this action. The data also indicate that elevated MDM2

levels; as seen in LNCaP-MST cells, result in a more pronounced apoptotic response to AS, and that under such conditions the addition of AD does not result in more apoptosis.

Overall Cell Death by Clonogenic Assay

Clonogenic survival experiments were performed to determine whether the added early cell killing from

TABLE III. Effect of Antisense MDM2 on LNCaP Cell Apoptosis Measured by the Annexin V-Assay

| Treatment | Mean | SEM | P* |
|---------------------------|------|-----|---------|
| Lipofectin control | 4.9 | 1.3 | — |
| AS (antisense MDM2) | 17.5 | 1.2 | 0.02 |
| ASM (mismatch control) | 8.3 | 0.9 | 0.264 |
| AD (androgen deprivation) | 8.5 | 1.6 | 1.000 |
| AD + AS** | 26.5 | 2.7 | <0.0001 |
| AD + ASM | 13.0 | 2.2 | 0.012 |
| AD + R1881 | 5.4 | 1.0 | 0.826 |
| AD + AS + R1881 | 15.2 | 4.2 | 0.174 |
| AD + ASM + R1881 | 8.5 | 2.1 | 1.000 |

LNCaP cells were treated for 48 hr with AS-MDM2 (200 nM) alone or in combination with AD \pm R1881 and the percentage of apoptotic cells measured by flow cytometric analysis of annexin V-PE and 7-AAD staining. The data shown represent the average values (\pm SEM) from three independent experiments.

*Compared to group above, one way Anova, Bonferroni test.

**Other comparisons: AD + AS versus AD + AS + R1881 ($P = 0.061$); AD + AS versus AS alone ($P = 0.32$).

TABLE IV. Effect of Antisense MDM2 on LNCaP-MST Cell Apoptosis Measured by the Annexin V-Assay

| Treatment | Mean | SEM | P* |
|---------------------------|------|-----|---------|
| Lipofectin control | 3.8 | 0.7 | — |
| AS (antisense MDM2) | 35.0 | 1.6 | <0.0001 |
| ASM (mismatch control) | 16.8 | 1.7 | 0.002 |
| AD (androgen deprivation) | 10.3 | 1.8 | 1.000 |
| AD + AS** | 35.3 | 3.6 | <0.0001 |
| AD + ASM | 21.8 | 3.6 | 0.035 |
| AD + R1881 | 7.3 | 1.3 | 0.018 |
| AD + AS + R1881 | 32.4 | 3.4 | <0.0001 |
| AD + ASM + R1881 | 20.0 | 2.0 | 0.073 |

LNCaP-MST cells were treated for 48 hr with AS-MDM2 (200 nM) alone or in combination with AD \pm R1881 and the percentage of apoptotic cells measured by flow cytometric analysis of annexin V-PE and 7-AAD staining. The data shown represent the average values (\pm SEM) from three independent experiments.

*Compared to group above, one way Anova, Bonferroni test.

**Other comparisons: AD + AS versus AD + AS + R1881 ($P = 1.000$); AD + AS versus AS alone ($P = 1.000$).

TABLE V. Effect of Antisense MDM2 on LNCaP Cell Caspase-3 + 7 Activity

| Treatment | Mean | SEM | P* |
|---------------------------|------|-----|---------|
| Lipofection control | 85 | 9 | — |
| AS (antisense MDM2) | 357 | 29 | <0.0001 |
| ASM (mismatch control) | 181 | 29 | 0.020 |
| AD (androgen deprivation) | 48 | 7 | 0.186 |
| AD + AS** | 635 | 64 | <0.0001 |
| AD + ASM | 121 | 26 | <0.0001 |
| AD + R1881 | 115 | 13 | 1.000 |
| AD + AS + R1881 | 430 | 36 | <0.0001 |
| AD + ASM + R1881 | 235 | 6 | 0.007 |

Caspase-3 + 7 activity was measured by fluorometric assay. LNCaP cells were cultured for 2 days with or without AD (\pm R1881) and then for 30 hr with 200 nM AS or ASM. The data shown represent the average values (\pm SEM) from three independent experiments.

*Compared to group above, one way Anova, Bonferroni test.

**Other comparisons: AD + AS versus AD + AS + R1881 ($P = 0.004$); AD + AS versus AS alone ($P < 0.0001$).

apoptosis due to AS + AD translates into a significant increase in overall cell killing. LNCaP and LNCaP-MST cells were cultured in CM, AD, or AD + R1881 medium for 2 days prior to exposure to AS for 24 hr before plating for clonogenic survival. As shown in Table VII, the percentages of LNCaP cells surviving were 59.7% for AS alone, 27.2% for AS + AD, and 54.7% for AS + AD + R1881. The difference between AS and AS + AD was significant. The percentage of LNCaP cells surviving after AS + AD + R1881 was reduced to

TABLE VI. Effect of Antisense MDM2 on LNCaP-MST Cell Caspase-3 + 7 Activity

| Treatment | Mean | SEM | P* |
|---------------------------|------|-----|---------|
| Lipofection control | 211 | 12 | — |
| AS (antisense MDM2) | 966 | 19 | <0.0001 |
| ASM (mismatch control) | 617 | 24 | 0.001 |
| AD (androgen deprivation) | 231 | 22 | <0.0001 |
| AD + AS** | 879 | 55 | <0.0001 |
| AD + ASM | 357 | 55 | <0.0001 |
| AD + R1881 | 234 | 4.9 | 1.000 |
| AD + AS + R1881 | 697 | 82 | <0.0001 |
| AD + ASM + R1881 | 345 | 41 | <0.0001 |

Caspase-3 + 7 activity was measured by fluorometric assay. LNCaP-MST cells were cultured for 2 days with or without AD (\pm R1881) and then for 30 hr with 200 nM AS or ASM. The data shown represent the average values (\pm SEM) from three independent experiments.

*Compared to group above, one way Anova, Bonferroni test.

**Other comparisons: AD + AS versus AD + AS + R1881 ($P = 0.251$); AD + AS versus AS alone ($P = 1.000$).

that seen with AS alone. For the LNCaP-MST cell line, no significant difference was observed between AS and AS + AD treatment.

DISCUSSION

Although AD is quite effective at reducing prostate cancer tumor burden and causing dormancy, an improvement in patient survival from AD treatment as a single agent has not been conclusively established. Early in the malignant transformation of prostate epithelial cells, the apoptotic response to AD is dramatically reduced or lost, and the principal response is a slowing of cell growth and reduction in cell proliferation [5–9]. Previously we found that R3327-G rat prostate tumors grown *in vivo* have a pronounced shift to quiescence in response to AD, with a minimal increase in apoptosis [3]. The human tumor LNCaP line responds similarly [27]. We hypothesized that if the apoptotic response of prostate cancer to AD could be restored through alterations in the molecular milieu, it may be possible to enhance overall tumor cell death. Such a strategy could then prolong clinical responses and perhaps ultimately patient survival. The oncogene MDM2 was selected as a target because it contributes significantly to the control of p53 function and preliminary western blot studies (not shown) revealed that MDM2 expression was tied to the apoptotic response of LNCaP cells to the combination of AD + RT.

MDM2 is a key protein in the apoptotic pathway that functions by negatively regulating p53 transcription directly and indirectly through effects on nuclear transport and p53 degradation. As a result, MDM2 interferes with normal growth control by preventing apoptosis [29]. Previous studies have shown that the suppression of MDM2 expression by AS increases the apoptotic response of some tumor cells to radiation or chemotherapy [15,17–21].

In the results presented here, AS specifically inhibited MDM2 expression in both LNCaP and MDM2 overexpressing LNCaP-MST cells. In parallel with this inhibition, p53 and p21 expression were enhanced. These increases were expected since MDM2 is a feedback regulator of p53. The incubation of cells in AD medium resulted in a decrease in MDM2 expression (LNCaP > LNCaP-MST). When the cells were cultured in AD medium with AS, there was a further abrogation of MDM2 expression in both cell lines. The suppression of MDM2 under the conditions of AS + AD resulted in proportionally increased tumor cell growth inhibition *in vitro* and significantly enhanced cell death responses (LNCaP > LNCaP-MST cells).

The major relative differences in the LNCaP and LNCaP-MST cell death responses were seen when

Lipofection

Small 12

TABLE VII. Effects of AS on LNCaP and LNCaP-MST Cell Clonogenic Survival When Cultured in Complete, Androgen Deprived (AD) or AD + R1881 Medium

| Treatment | % Clonogenic survival | | | |
|------------------|-----------------------|---------|-----------------|-------|
| | LNCaP | | LNCaP-MST | |
| | M \pm SEM | P* | M \pm SEM | P* |
| ASM | 85.4 \pm 0.9 | — | 88.7 \pm 2.4 | — |
| AS | 59.7 \pm 7.5 | 0.015 | 67.6 \pm 9.4 | 1.000 |
| AD + ASM | 64.2 \pm 3.0 | 1.000 | 70.9 \pm 7.0 | 1.000 |
| AD + AS** | 27.2 \pm 5.7 | 0.001 | 55.0 \pm 11.2 | 1.000 |
| AD + ASM + R1881 | 77.8 \pm 1.1 | <0.0001 | 83.5 \pm 3.1 | 0.518 |
| AD + AS + R1881 | 54.7 \pm 2.6 | 0.033 | 61.5 \pm 12.4 | 1.000 |

*Compared to group above, one-way Anova, Bonferroni test, done separately for the six LNCaP and six LNCaP-MST groups. The data shown represent the mean (M) values (\pm SEM) from three independent experiments.

**Other LNCaP comparisons (n = 6 groups): AD + AS versus AD + AS + R1881 ($P = 0.009$); AD + AS versus AS alone ($P < 0.002$). Other LNCaP-MST comparisons (n = 6 groups): AD + AS versus AD + AS + R1881 ($P = 1.000$); AD + AS versus AS alone ($P = 1.000$).

AS was combined with AD. There was little disparity after treatment with lipofectin or AD alone in trypan blue uptake or apoptosis between LNCaP and LNCaP-MST cells. However, the LNCaP and LNCaP-MST lines responded differently to AS, AS + AD, and AS + AD + R1881. Overall early cell death by trypan blue uptake in LNCaP-MST cells incubated with AS + AD was greater than AS alone (Table II), but was half that seen in LNCaP cells (Table I). In contrast, the apoptotic response of LNCaP-MST cells to AS alone was greater than that of LNCaP cells; yet, there was no additional cell death from the combination of AS + AD. Wild type LNCaP cells had less of an apoptotic response to AS alone and a supra-additive response to AS + AD (Tables III–VI).

The discrepancy in the features of the trypan blue and apoptotic responses of LNCaP-MST cells to AS and AS + AD may be related to the intrinsic differences in the assays. Trypan blue uptake was measured over 3 days and included death from necrosis and apoptosis. The apoptosis assays were run over a shorter period of time and represent more of a snapshot of this form of cell death. Since cell death is a dynamic process, wherein cell death and disintegration may not be constant over time, some variation between assays is expected. Nonetheless, the early high apoptotic response of LNCaP-MST cells to AS and the lack of any added effect from AD was notable; this pattern was confirmed in both the annexin V and caspase-3 + 7 assays. One explanation is that the condition of MDM2 overexpression, which negatively affected p53 expression, created an enhanced sensitivity to MDM2 suppression by AS, resulting in higher levels of apoptosis than that seen in wild type LNCaP cells.

When the synthetic androgen R1881 was added back to the AD medium and LNCaP cells exposed to AS (AS + AD + R1881), cell death was reduced to approximately the levels seen with AS alone. The supra-additive cell death from AS + AD was clearly related to an interaction that appeared to result in the restoration of the apoptotic response to AD. More importantly, the same supra-additive effect was observed by clonogenic survival assay.

Wild type LNCaP cells displayed a supra-additive reduction in clonogenic survival to AS + AD, when compared to AS or AD given individually. The effect of AS + AD was reversed by adding R1881. Such a supra-additive decrement in clonogenic survival was not seen with LNCaP-MST cells. Thus, the blunted response of LNCaP-MST cells to AS + AD over that of AS alone was observed in both apoptotic (Tables IV and VI) and clonogenic survival (Table VII) assays; although, a significant increase in cell death from AS + AD over AS alone was seen for trypan blue dye uptake (Table II). As mentioned above, the trypan blue uptake and apoptosis assays measure early cell death response. Clonogenic survival sums cell death effects over time and is the more important assay. Thus, MDM2 proved to be central to the response of both cell lines to AD. MDM2 suppression in the setting of wild type LNCaP cells appeared to restore the cell death response to AD that was lost during transformation. MDM2 overexpression in LNCaP-MST cells resulted in a significant reduction in the interaction of AS with AD, confirming that MDM2 is an important determinant of the cell death response of prostate cancer cells to AD.

The molecular mechanisms that govern the response of androgen sensitive prostate cancer cells to AD alone

are unclear. There is primarily indirect evidence that abnormal p53 expression may contribute to the development of androgen insensitivity; this evidence is derived mainly from analyses of tumor tissue from patients with androgen insensitive prostate cancer [24,25,30]. Burchardt et al. [26] have shown more directly that the suppression of p53 function, using antisense p53, resulted in LNCaP tumor cell growth in castrated nude mice that did not occur when p53 was expressed. In addition, there are data suggesting that p21 is an important determinant of prostate cancer androgen insensitivity [31–33]. Antisense MDM2 has a downstream effect on p21 through p53. Recent data also suggest that antisense MDM2 promotes apoptosis in vitro and tumor growth inhibition in vivo independent of p53 status [15,34,35]. Thus, MDM2 appears to have p53 dependent and independent pathways that promote tumor resistance to treatments such as radiation, chemotherapy, and as shown in this report, AD.

In conclusion, our data reveal for the first time that MDM2 expression has a key role in the early (apoptotic) and overall (clonogenic) cell death response of prostate cancer to AD. The supra-additive cell killing from AS+AD was reversed by androgen replacement, suggesting that AS restored the apoptotic response of LNCaP cells. The clinical implications are considerable. Antisense MDM2 should impact on the full spectrum of patients with prostate cancer. Patients with advanced metastatic disease would have more pronounced and probably lasting responses to AD. Intermediate and high risk prostate cancer patients with clinically localized (no overt evidence of metastasis) stand to benefit from more complete eradication of micrometastatic disease from the addition of AS to sensitize cells to AD and local disease from the addition of AS to sensitize cells to radiation. Patients with early disease treated with radiotherapy might gain from the use of AS to lower radiation doses needed for cure. The application of AS to the combination AD+RT is currently under investigation.

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